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Licenciada em Bioquímica

Optimization of FucoPol bioreactor production and exopolysaccharide applications

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Doutora Maria Filomena Andrade de Freitas, Senior Researcher, FCT-UNL

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Palavras-chave
FucoPol, exopolissacárido, *Enterobacter* A47, impacto do fósforo, atividade biofloculante, bioadsorção de metais pesados.

Resumo

O FucoPol é um exopolisacárido (EPS) produzido pela bactéria *Enterobacter* A47, composto por açúcares (fucose, galactose, glucose e ácido glucurónico) e grupos acilo (sucinato, piruvato e acetato), e possui um peso elevado molecular de (>10^6 Da).

O primeiro objetivo desta tese foi investigar o efeito da concentração de fósforo (P) na produção deste polissacárido. Para tal, foram realizados ensaios de produção com diferentes concentrações deste nutriente: de 0.8 a 2.6 $g.L^{-1}$ (concentração standard). As condições implementadas não afetaram o crescimento da *Enterobacter* A47, mas resultaram numa redução da produção de EPS: 24% menos polímero quando 2 e 1.3 $g.L^{-1}$ de P foram utilizadas, e 44% para 0.8 $g.L^{-1}$ de fósforo. Uma pequena redução na concentração de fósforo para 2.0 $g.L^{-1}$ não surtiu efeito na composição do polímero, nem no peso molecular. Contudo, os EPS obtidos nos ensaios com menos P apresentaram menor conteúdo em fucose e eram ricos em glucose.

O segundo objetivo deste trabalho foi avaliar as propriedades floculantes do FucoPol. A taxa de floculação foi determinada utilizando uma suspensão de pó caulim (5 $g.L^{-1}$) na presença de $CaCl_2$. Pequenas quantidades de biofloculante (1 $mg.L^{-1}$) apresentaram taxas de floculação superiores a 70% para valores de pH entre 3-5 e temperaturas entre 15-20 ºC. O biofloculante também se apresentou estável após ser submetido a congelamento/descongelamento e aquecimento até 100 ºC, durante 20 min.

O trabalho realizado nesta tese também teve como objetivo explorar a capacidade de o polissacárido adsorver metais pesados. O FucoPol revelou um bom desempenho na bioadsorção de cobalto, cobre e zinco. Este polímero foi excecionalmente eficiente na remoção de chumbo, pelo que este metal foi escolhido para estudos acerca do impacto de diferentes fatores, nomeadamente da concentração de EPS, da concentração inicial de Pb^{2+}, pH e temperatura. À concentração ótima (5 $mg.L^{-1}$), o FucoPol demonstrou ser capaz de adsorver até 18645 $mg_{metal}.g^{-1}_{EPS}$ de uma solução com 100 $mg.L^{-1}$ de Pb^{2+}, a pH 2.3. Este polímero também mostrou ser eficaz a adsorver metal numa gama de temperaturas entre 5 e 45 ºC.

Keywords
FucoPol, exopolysaccharide, *Enterobacter* A47, phosphorus impact, bioflocculant activity, heavy metal biosorption.

Abstract

FucoPol is an exopolysaccharide (EPS) produced by the bacterium *Enterobacter* A47, composed of sugars (fucose, galactose, glucose and glucuronic acid) and acyl groups (succinyl, pyruvyl and acetyl), and with a high average molecular weight (>10^6 Da).

The first main goal of this thesis was to investigate the effect of the phosphorus (P) concentration on FucoPol production. Production assays were performed under different concentrations of this nutrient: from 0.8 to 2.6 $g.L^{-1}$(standard concentration). The conditions implemented did not affect *Enterobacter* A47 growth, but a reduction of the EPS synthesis was observed: 24% less polymer when 2 and 1.3 $g.L^{-1}$ of P were used, and 44% for 0.8 $g.L^{-1}$ of phosphorus. A slight reduction to 2.0 $g.L^{-1}$ in the phosphorus concentration did not affect the polymer's composition, neither the molecular weight. However, the EPS obtained from the assays with fewer P showed less fucose content and was richer in glucose.

Secondly, this thesis aimed to assess the flocculation properties of FucoPol. The flocculation rate of FucoPol was determined using a kaolin clay suspension (5 $g.L^{-1}$) in the presence of $CaCl_2$. Flocculation rate values above 70% were achieved with a low bioflocculant dosage of 1 $mg.L^{-1}$, for pH values in the range 3-5, and temperature within 15-20 ºC. The bioflocculant was also shown to be stable after freezing/thawing and heating up to 100 ºC, for 20 min.

The work performed in this thesis also had as an objective to explore the metal binding efficiency of the polysaccharide. FucoPol revealed a good performance in the biosorption of cobalt, copper and zinc. The polysaccharide was an excellent biosorbent of lead so this metal was chosen for further studies, namely the impact of EPS dosage, Pb^{2+} initial concentration, pH and temperature. Optimal FucoPol concentration of 5 $mg.L^{-1}$ was found to uptake 18645 $mg_{metal}.g^{-1}_{EPS}$ from an 100 $mg.L^{-1}$ Pb^{2+} solution, at pH 2.3. Moreover, FucoPol presented a great sorption performance in the range of temperatures between 5 and 45 ºC.

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Abbreviations

ATP	Adenine triphosphate
CDW	Cell Dry Weight
C/N	Carbon to nitrogen ratio
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EPS	Exopolysaccharide
FR	Flocculation rate
GDP	Guanidine diphosphate
HPLC	High performance liquid chromatography
IM	Inner membrane
IR	Infrared
LB	Luria broth
N. A.	Data not available
NDP	Nucleotide diphosphate
NH_4^+	Ammonia
NMWCO	Nominal molecular weight cut-off
OD	Optical density
OM	Outer membrane
P	Phosphorus
PDI	Polydispersity índex
Pi	Inorganic phosphate
Poly P	Polyphosphate
Pr	Protein

PS	Polysaccharide
SEC-MALLS	Size Exclusion Chromatography – Multi-Angle Laser Light Scattering
SEM	Scanning electron microscopy
SLPB	Standard liters per minute
TFA	Trifluoroacetic acid
UDP	Uridine diphosphate

Variables

C_{metal}	Metal concentration after equilibrium ($mg.L^{-1}$)
dP	Variation of concentration of product ($g.\ L^{-1}$)
dt	Variation of time (days)
M	Metal adsorbed to the polysaccharide (mg_{metal})
m_{EPS}	Exopolysaccharide weight (mg)
M_n	Number average molecular weight
M_w	Average molecular weight
q	Metal uptake ($mg_{metal}.g_{EPS}^{-1}$)
r_P	Volumetric productivity ($g.L^{-1}.d^{-1}$)
V	Volume (L)
$Y_{p/S}$	Product yield on substrate ($g.g^{-1}$)
Δp	Product produced (g_{EPS})
ΔS	Substrate consumed ($g_{glycerol}$)

1. Introduction and Motivation

1.1. Polysaccharides

Polysaccharides are widely used in several growing industries, such as the pharmaceutical, cosmetic, food and paper industries, as well as in oil recovery and water treatment processes (Alves et al., 2010a; Freitas et al., 2011a). These materials are commercially appealing due to their interesting physical and structural properties (Cruz et al., 2011), since they can be used as emulsifiers, viscosifiers, stabilizers, thickening or flocculating agents (Alves et al., 2010a; Cruz et al., 2011). Moreover, the use of polysaccharides obtained from natural sources instead of synthetic polymers reduces the negative impact of those materials on the environment, since they are biodegradable and non-toxic (Ferreira et al., 2014). Despite the fact that these biopolymers can be obtained from several organisms (plants, algae and animals), their availability is largely influenced by uncontrollable conditions, such as climate and pollution (Alves et al., 2010a). Nevertheless, numerous bacteria have the ability to synthesize biopolymers which represent a more advantageous process in obtaining polysaccharides, since microorganisms have higher growth rates and the production conditions can be easily optimized, improving productivity and the polymer's desirable characteristics (Alves et al., 2010a; Cruz et al., 2011). Moreover, agro-industrial byproducts or wastes, such as glycerol (biodiesel byproduct), can be used as a carbon source, making the use of these microorganisms an economic viable alternative (Donot et al., 2012).

Depending on their biological function, bacterial polysaccharides have different cellular locations: they can either be intracellular, associated with the storage of nutrients (e.g. glycogen), extracellular (e.g. xanthan), or as a compound of the cell wall (e.g. peptidoglycans), granting protection and structure to the cell (Schmid et al., 2015a). Extracellular polysaccharides or exopolysaccharides (EPS), are found outside the cell wall, either forming the bacterial capsule (covalently bound to the cell membrane) or as a slime which is loosely bound to the cell surface (Nwodo et al., 2012a; Suresh Kumar et al., 2007a). As such, harvesting the EPSs that were exported to the extracellular environment is rather easy (Schmid et al., 2015a), making EPSs valuable alternatives in the biopolymers' market.

Due to this fact, bacterial exopolysaccharides have been increasingly studied and new EPS have been reported in recent years. For example, xanthan, one of the most significant industrial microbial biopolymers, was found to be produced by the bacterium *Xanthomonas campestris* in the 1950s (Garcıa-Ochoa et al., 2000). Nowadays, xanthan gum is used in oil recovery, food and pharmaceutical industries due to its rheological properties, such as high viscosity and stability

over a wide range of conditions (pH, temperature, salt concentration) (Rosalam and England, 2006). Gram-negative bacteria of the genus *Sphingomonas* were found to produce different biopolymers (known as sphingans), such as gellan, welan, rhamsan or diutan. Gellan is mainly used as thickener and suspending agent in the medical and food industries since it is a gelling agent and provides stability to temperature and pH (Fialho et al., 2008). Alginate was first discovered in brown seaweeds in the 1880s and, later, it was found that the species *Pseudomonas aeruginosa* and *Azotobacter vinelandii* were able to produce it as well. The use of this EPS has increased in recent years, since it can not only be used in the food and paper industries, but also, due to its biocompatibility, in the pharmaceutical and medical industries: in surgical and wound dressings, in controlled drug release and as dental impression compounds (Hay et al., 2013). Also, glucose-containing homopolysaccharides have found several applications in different areas. Examples include: dextran, produced by the bacterium *Leuconostoc mesenteriodes*, that is used as chromatographic media and in the food and pharmaceutical industries; curdlan, isolated from *Rhizobium meliloti* and *Agrobacterium radiobacter*, has been applied in the removal of heavy metals and in the food and pharmaceutical fields. In addition, cellulose, a basic structural biomaterial found in plants, can also be obtained from *Acetobacter* spp. and is used in food (as an ingestible fiber) and in the biomedical field (Nwodo et al., 2012a).

1.2. Composition of Exopolysaccharides

Polysaccharides are high molecular weight molecules (10^4 to 10^7 Da), obtained by polymerization of several sugar monomers, wherein glucose and galactose are the most common residues (Sutherland, 2001). These monomers are usually neutral sugars, acidic sugars (e.g. uronic acid) and/or amino-sugars (Torres et al., 2011), though, frequently, polysaccharides also contain non-sugar elements, namely organic acyl groups (e.g. acetate, pyruvate) and inorganic compounds (e.g. phosphate, sulphate) (Jaiswal et al., 2014; Nwodo et al., 2012a).

In terms of their chemical composition, these molecules can be classified as homopolysaccharides (e.g. levan, dextran, curdlan), composed of only one type of monosaccharide; or heteropolysaccharides (e.g. alginate, xanthan, gellan), composed of repeating units, varying in size from disaccharides to octasaccharides, with different types of monosaccharides in their composition (Nwodo et al., 2012a; Sutherland, 2001). Heteropolysaccharides also have non-carbohydrate substituents, such as pyruvate, acetate esters, succinates, phosphates, sulphates or uronic acids, some of which grant an anionic character to those macromolecules. These different charged substituents are responsible for the adsorption sites found in EPSs that allow interactions with ions and other molecules, making exopolysaccharides very useful in several applications (More et al., 2014; Sutherland, 2001).

1.3. EPS: biological functions and biosynthesis

Exopolysaccharides have different physiological roles depending on the microorganism and the environmental circumstances (Donot et al., 2012). Although EPSs are majorly associated with protective mechanisms, since they can prevent cellular desiccation due to their water retention capacity, and protect against predatory microorganisms (Suresh Kumar et al., 2007a), these biopolymers also act as structural elements in the formation of biofilms and play a major role in the adherence to surfaces (More et al., 2014). They are important in interactions with other microorganisms, namely in the bacterial cell-cell recognition and aggregation processes, and are also responsible for the sorption of exogenous compounds and inorganic ions, due to their anionic charge (Donot et al., 2012; More et al., 2014; Whitney and Howell, 2013)

These biopolymers can be synthesized by two different mechanisms depending on the bacteria that produces them. EPS originated from gram-positive bacteria are synthesized in the exterior of the cell by enzymes that were secreted by the bacteria or that are anchored to the cell surface (e.g. levan, dextrans and alternans). On the other hand, gram-negative bacteria synthesize EPSs intracellularly and then secrete the polysaccharides to the extracellular environment (e.g. xanthan, gellan, cellulose) (Madhuri and Prabhakar, 2014; Sutherland, 2001).

For gram-negative bacteria, the bioprocess can be divided into 3 steps that are mediated by different enzymes: EPS biosynthesis starts in the cytoplasm with the activation of simple sugars through the phosphorylation of monosaccharides (fig 1.1), followed by polymerization of the EPS and, finally, the secretion of the exopolysaccharide to the external environment.

In order to be used by the cell, sugar molecules are obtained from the substrate and phosphorylated to sugar-6-phosphate (e.g. glucose-6-phosphate). In the EPS biosynthesis pathways, this molecule is a substrate for a second enzyme, phosphoglucomutase, responsible for the formation of sugar-1-phosphate (e.g. glucose-1-phosphate). These monosaccharides are important intermediates in the production of sugar nucleotides, such as uridine diphosphate glucose (UDP-Glucose), which is obtained using glucose-1-phosphate in a reaction catalyzed by uridine diphosphate-glucose pyrophosphorylase. At this stage, intracellular enzymes catalyze the interconversion of UDP-Glucose into other sugars (e.g. UDP-Glucuronic acid), creating diverse sugar monosaccharides. Next, periplasmatic enzymes (glycosyltransferases) catalyze the transfer of the several activated sugar precursors and the acyl groups (if present) onto a glycosyl transporter lipid, an isoprenoid alcohol (Madhuri and Prabhakar, 2014; Suresh Kumar et al., 2007b). This carrier is anchored to the cell membrane and improves the correct assembly of the exopolysaccharide subunits (Donot et al., 2012). Afterwards, the assembled polymer is excreted

to the exterior of the cell by one of two possible mechanisms, Wzx/Wzy- or ABC transporter-dependent pathways (Schmid et al., 2015b; Whitney and Howell, 2013).

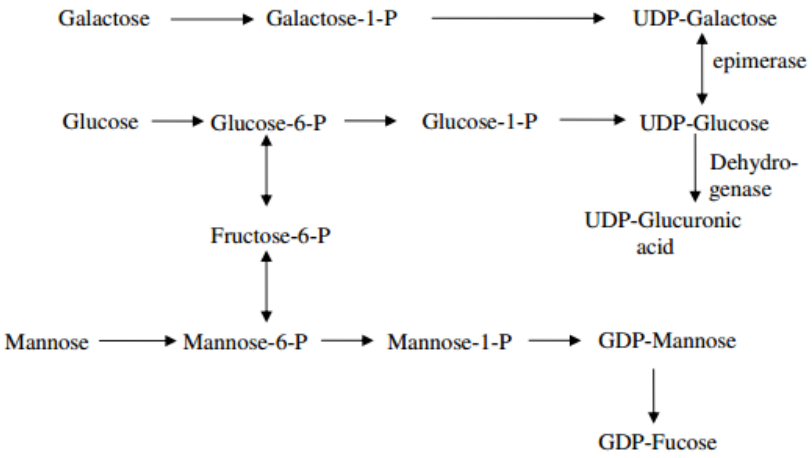

Figure 1.1 – Schematic representation of the metabolic pathways associated with the synthesis of nucleoside sugars that are involved in the biosynthesis of exopolysaccharides in gram-negative bacteria (reproduced with permission from Suresh Kumar et al., 2007).

1.4. FucoPol

In addition to their valuable properties, some EPS possess rare sugar monomers in their composition, which increases their market value. One of those monosaccharides that are rarely found in Nature is L-fucose (Roca et al., 2015). Moreover, it was reported that fucose-containing polysaccharides have biological activity, since fucose is able to reduce allergic reactions, accelerate wound healing (Péterszegi et al., 2003), also shows an anti-aging effect (Fodil-Bourahla et al., 2003), and can be used as a skin moisturizer or as an anti-cancer and anti-inflammatory agent (Cescutti et al., 2005). These properties make fucose-containing biopolymers suitable to be used in the therapeutic and cosmetic areas.

Fucose-containing biopolymers are produced by numerous bacterial genera, as several studies have documented: *Clavibacter michiganensis* exhibits the production of an EPS rich in fucose named clavan; *Klebsiella pneumonia* I-1507 is responsible for the production of fucogel used in the cosmetic industry; and some members of the family *Enterobacteriaceae* are able to synthesize colanic acid, a fucose-containing exopolysaccharide, as well (Freitas et al., 2011b).

Furthermore, the gram-negative bacterium *Enterobacter* A47 (DSM 23139)[1] was previously reported to produce a high molecular fucose-containing EPS, which was named FucoPol (Alves et al., 2010b). FucoPol is a heteropolysaccharide composed of sugar residues: fucose (32-36%mol), galactose (25-26%mol), glucose (28-37%mol) and glucuronic acid (9-10%mol); and acyl groups: succinyl (2-3%wt.), pyruvyl (13-14%wt.) and acetyl (3-5%wt.) (Torres et al., 2015). The composition of FucoPol varies depending on the physicochemical factors of the cultivation conditions, which makes this process very versatile, since it can originate different polysaccharide products that can be used in different applications (Freitas et al., 2014).

1.5. Motivation

As mentioned above, bacterial exopolysaccharides are biocompatible, non-toxic and eco-friendly macromolecules that can be easily obtained from renewable sources. Moreover, EPSs obtained from microorganisms have different structural composition, showing a high variety of interesting properties: these biopolymers can change the rheological behavior of solutions and show great adsorption abilities (More et al., 2014).

Despite their useful properties, costs of production are still high, which difficult the commercialization of these microbial products. In order to reduce the costs associated to the process, exopolysaccharide production can be optimized either by manipulating the environmental conditions (pH, temperature), or by optimizing the nutritional requirements (carbon, nitrogen, phosphorus). As so, the first objective of this master's thesis was to optimize the production of FucoPol, a fucose-containing exopolysaccharide produced by *Enterobacter* A47 (Alves et al., 2010b). Several cultivation parameters, such as the pH, temperature and the carbon/nitrogen ratio, were optimized in previous studies (Torres et al., 2012, 2014). This work aimed to assess the effect of phosphorus concentration on FucoPol production, as well as the impact on the polymer's composition and molecular mass distribution.

Additionally, the suitability of FucoPol to be used as a flocculating agent and for the removal of heavy metals was evaluated.

[1] Enterobacter A47: access number DSM 23139 at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

2. Effect of phosphorus concentration on FucoPol production

2.1. Introduction

Over the past decade, attention has been raised towards several novel microbial extracellular polysaccharides (EPS) with industrially interesting properties that make them suitable to be used as biomaterials in the most different areas, such as medical, cosmetic, pharmaceutical, environmental or even in oil-recovery and paper industries (Roca et al., 2015). Despite their potential, the majority of EPS production processes are not economically viable to compete with polymers from other sources that currently dominate the market (Freitas et al., 2011a). Hence, a better understanding of the different factors that have an impact in the synthesis of EPS is needed in order to optimize the process and minimize the costs of production (Rehm, 2010).

EPS-producing organisms use a diverse range of carbon and nitrogen sources, and have different nutritional and environmental needs. In fact, the yield and efficiency of the EPS production process is largely influenced by several physicochemical factors, such as the carbon/nitrogen ratio (C/N), temperature, pH and nutrients concentration (Nwodo et al., 2012). EPS production frequently occurs under aerobic conditions and usually increases under sub-optimal incubation temperatures and constant neutral pH (Suresh Kumar et al., 2007).

As for the carbon sources, sugars, such as sucrose, glucose, lactose and maltose, are the most commonly used (Suresh Kumar et al., 2007). However, in order to reduce production costs, agro-industrial wastes and byproducts, such as glycerol, cheese whey, molasses or starch, are increasingly being used as carbon sources for exopolysaccharide production (Rehm, 2010; Roca et al., 2015). On the other hand, the use of those less pure substrates can be difficult to implement if the final product is going to be used in applications where high-purity and quality are required (Roca et al., 2015).

For the majority of these fermentative production processes, the yield is maximized with an excess of carbon source and a limitation of other nutrients, such as nitrogen, oxygen or phosphorus (Freitas et al., 2011a). In fact, it is known that FucoPol synthesis occurs under carbon availability concomitant with nitrogen and oxygen limitation (Torres et al., 2015), but the effect of phosphorus concentration hasn't been studied in great detail.

Phosphorus (P) is an essential nutrient for all living organisms, as it has a key role in several biological processes. Moreover, P is also present in the composition of several important

macromolecules and cellular compounds: in cellular membranes (phospholipids), in the DNA that contain all the genetic information of the cell, in the RNA, and also in the main molecule responsible for energy-transfer in the cell, ATP (White and Metcalf, 2007). Furthermore, inorganic phosphate (Pi) is involved in intracellular signaling in the regulation of several cellular processes, such as membrane transport, enzyme activity and in the metabolic synthesis of macromolecules (Santos-Beneit, 2015).

Despite this nutrient's importance in the life of the cell, phosphorus availability in the environment is low (Santos-Beneit, 2015). So, researchers have been focusing in understanding the mechanisms involved in the phosphorus uptake process in bacteria. Gram-negative bacteria, such as *Escherichia coli* and *Enterobacter* sp., have an outer membrane (OM), that separates de external environment from the periplasm; and a cytoplasmatic or inner membrane (IM), that allows the concentration of nutrients in the cytoplasm (Beveridge, 1999; Rao and Torriani, 1990). Phosphate, like other nutrients, is able to cross the OM into the periplasm through channels (pores) that facilitate the diffusion of these small molecules. These phosphorylated compounds are then degraded by enzymes to inorganic phosphate (Pi) and captured by proteins that transport the Pi across the cytoplasmatic membrane (Rao and Torriani, 1990). There are two transport systems responsible for the uptake of phosphorus compounds by the cell: the Pst system, which is repressed by high Pi concentrations; and the Pit system, which is fully constitutive. In optimal nutritional conditions, the Pit transport system supplies P to the cell at the same rate as it is metabolized, also controlling the excretion of the excessive Pi out of the cell (Jansson, 1988). Phosphorus-starvation conditions induce the transcription of the Pho regulon that codes for several proteins involved in the transport of P into the cell and, therefore, activate the Pst transport system (Rao and Torriani, 1990; Santos-Beneit, 2015). In this conditions, a specific transporter (Ugp system) is activated and glycerol-phosphate is transported across the IM in order to maintain the optimal Pi concentration in the cell (Brzoska et al., 1994).

The majority of the metabolic precursors used in the cell biosynthetic pathways are phosphorylated (Rao and Torriani, 1990) and exopolysaccharide synthesis is no exception. In fact, the first step in the EPS biosynthesis process is the phosphorylation of the carbon source to sugar-6-phosphate (see section 1.3).

As so, the scientific community is interested in further understanding the impact of the phosphorus content in the fermentation process of EPS production. Souw and Demain (1979) proved that the production of xanthan was controlled by the level of P in the medium. Indeed, this study showed that high concentrations of this nutrient in the medium inhibited the synthesis of this polymer. Clementi et al. (1995) proved that the cultivation of *Azotobacter vinelandii* under phosphorus-limited media increased the yield of alginate production. Previous studies have also

shown that for many EPS-producers, the phosphorus concentration that maximizes cell growth was not the same that optimized the production of EPS, such as the production of gellan and xanthan (Lee et al., 2009; Souw and Demain, 1979; Umashankar et al., 1996). On the other hand, a nutritional study performed with *Klebsiella pneumoniae* concluded that a reduction in the P content in the medium enhanced the production of a rhamnose-containing polysaccharide (Farres et al., 1997). Also, a study performed by Mendrygal and González (2000) showed that the phosphorus concentration determines which polysaccharide is produced by *Sinorhizobium meliloti*, since low P concentration increased the synthesis of one type of polymer, the EPS II, while high phosphorus in the medium stimulated the production of succinoglycan. These authors were also able to relate the production of EPS II with a protein that is known to be involved in the regulation of the phosphate uptake in *Escherichia coli*, pho B (Jansson, 1988; Mendrygal and González, 2000). Janczarek and Urbanik-Sypniewska (2013) found that the Pi concentration affected the transcription of a gene required for the exopolysaccharide synthesis by *Rhizobium leguminosarum* and that phoB was one of the proteins responsible for the regulation of this gene.

This work evaluated the impact of phosphorus concentration on the production and composition of the EPS produced by *Enterobacter* A47.

2.2. Materials and Methods

2.2.1. Biopolymer Production

2.2.1.1. Cultivation media

The pre-inocula was performed in 250 mL baffled shake flasks with 50 mL of Luria broth (LB) medium (pH 7), which had the following composition (per liter): peptone, 10.0 g; yeast extract, 5.0 g; and NaCl, 10.0 g.

For the inocula, 500 mL Erlenmeyers with 200 mL of Medium E* (pH 7) were prepared with the following composition (per liter): $(NH_4)_2HPO_4$, 3.3 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g; 10 mL of a 100 mM $MgSO_4$ solution; and 1 mL of a micronutrient solution. This solution was composed (per liter of 1 N HCl): $FeSO_4 \cdot 7H_2O$, 2.78 g; $MnCl_2 \cdot 4H_2O$, 1.98 g; $CoSO_4 \cdot 7H_2O$, 2.81 g; $CaC_{l2} \cdot 2H_2O$, 1.67 g; $CuCl_2 \cdot 2H_2O$, 0.17 g; and $ZnSO_4 \cdot 7H_2O$, 0.29 g (Freitas et al., 2014). Medium E* was then supplemented with ~ 40 $g.L^{-1}$ of glycerol (ReagentPlus 86-88% w/w, Scharlau) as a carbon source.

All solutions used were previously autoclaved (20 minutes at 120 ºC, 1 bar) and all microorganism manipulations were performed in a laminar flow chamber, ensuring the maintenance of sterile conditions.

2.2.1.2. Microorganism

FucoPol was produced by *Enterobacter* A47 (DSM 23139), which was preserved in glycerol (20% v/v) at -80ºC. Culture reactivation was performed by growing the stock cultures in an agar plate (CHROMagar™ Orientation) for 24 h, at 30 ºC. Afterwards, pre-inocula were prepared by inoculating an isolated colony from the plate into 50 mL of Luria broth (LB) medium and incubation for 24 h in an orbital shaker (New Brunswick Scientific), at 30 ºC and 200 rpm. In order to prepare the inocula, Medium E* (200 mL) was inoculated with 20 mL of the pre-inocula and incubated, during 72 h under the same conditions.

2.2.1.3. Bioreactor Operation

In order to explore the effect of phosphorus in the production of FucoPol, different concentrations of phosphorus where tested: 2.6 $g.L^{-1}$ (the standard phosphorus concentration in Medium E*), 2.0 $g.L^{-1}$, 1.3 $g.L^{-1}$ and 0.8 $g.L^{-1}$.

All assays were performed in a 2 L bioreactor (BioStat B-plus, Sartorius, Germany), using a modified Medium E*, differing in the phosphorus concentration, supplemented with glycerol (40 $g.L^{-1}$). The temperature was maintained at 30.0 ± 0.1 ºC and the pH was automatically controlled at 6.98 ± 0.05, by the automatic addition of NaOH (5 M) and HCl (2 M). A silicon-based antifoam (Sigma-Aldrich, Germany) was used in order to prevent the formation of foam throughout the process.

The bioreactor was inoculated aseptically with 10% (v/v) inocula and operated in batch mode during the first 10 hours after inoculation and, afterwards, in a fed-batch mode, wherein a feeding solution (modified Medium E*, previously supplemented with 200 $g.L^{-1}$ of glycerol) was supplied to the reactor at a constant rate of 5 $mL.h^{-1}$.

The air flow rate (0.4 SLPM, standard liters per minute, of compressed air) was kept constant during the cultivation and the dissolved oxygen concentration (DO) was controlled at 10% of the air saturation by automatic variation of the stirrer speed (300-800 rpm) provided by two 6-blade impellers (Freitas et al., 2011b, 2014).

Culture broth samples (24 mL) were periodically recovered from the bioreactor throughout the experiments for measurement of the culture's broth apparent viscosity, quantification of nutrients' consumption and production of biomass and polysaccharide. Cultivation broth samples were centrifuged at 10956 x g, for 10 min. (Sigma 4-16 KS, Germany) in order to separate the biomass from the cell-free supernatant. Viscous samples were diluted with deionized water (dilutions of 1:2, 1:4 or 1:10, v/v, depending on the viscosity) before centrifugation, for viscosity reduction. The cell-free supernatant was preserved at -20 ºC and was used to determine the glycerol, ammonium and phosphate concentration, as well as to quantify the EPS production throughout the assays. The cell pellets were washed with deionized water and freeze dried (Scanvac, CoolSafe) for 48 hours, for quantification of phosphorus content in the biomass.

2.2.2. Analytical techniques

2.2.2.1. Apparent viscosity

The apparent viscosity of the cultivation broth samples was measured using a digital viscometer (FungiLab Alpha Series, Spain) in order to evaluate the EPS production during the assay. The viscosity, in centipoise (cP), was measured at different rotational speeds in the interval of 100 to 0.3 rpm. For highly viscous samples, the apparent viscosity was measured using a

controlled stress rheometer (HAAKE MARSIII, Thermo Scientific) equipped with a cone-plate geometry (diameter 35 mm, angle 2º), with a gap of 0.105 mm. The samples were equilibrated at 25 ºC, for 5 min, after which the flow curves were obtained using a steady-state flow ramp in the range of shear rate from 10^{-5} to 1000 s^{-1}.

2.2.2.2. Determination of cell growth

Culture growth was determined by measuring the absorbance at 450 nm (with a VWR V-1200 spectrophotometer, Portugal) of the broth samples acquired throughout the cultivation run. The cell dry weight (CDW) was calculated considering that one unit of OD_{450nm} is equivalent to 0.26 g L^{-1} CDW (as determined in previous studies). This analysis was performed in duplicate.

2.2.2.3. Glycerol concentration

Glycerol concentration in the cell-free supernatant was determined by high performance liquid chromatography (HPLC) with a VARIAN Metacarb column (BioRad) coupled to an infrared (IR) detector. The analysis was performed at 50 ºC, using H_2SO_4 (0.01 N) as eluent at a flow rate of 0.6 mL.min^{-1}. The samples were diluted (1:50) with a solution of H_2SO_4 (0.01 N) and filtered using Vectra Spin Micro Polysulfone filters (Whatman), which had a pore diameter of 0.2 μm, at 3000 rpm for 10 min. A standard calibration curve was constructed by preparing solutions with different glycerol (ReagentPlus 86-88% w/w, Scharlau) concentrations: 1.0 g.L^{-1}, 0.5 g.L^{-1}, 0.25 g.L^{-1}, 0.125 g.L^{-1} and 0.0625 g.L^{-1} (calibration curves in appendix 7.1). This analysis was performed in duplicate.

2.2.2.4. Ammonia and phosphorus concentration

Phosphorus and ammonia concentration in the cell-free supernatant (dilution 1:200) were determined by colorimetry using a flow segmented analyzer (Skalar 5100, Skalar Analytical, The Netherlands). Standard solutions of phosphorus (KH_2PO_4) and ammonia (NH_4Cl) were also prepared (4 – 20 ppm). This analysis was performed in duplicate.

For determination of the phosphorus content in the biomass, the freeze dried pellets were weighted (approximately 5 mg) and submitted to an acid digestion: the samples were hydrolyzed (1 h at 100 ºC) with 400 mg of $K_2S_2O_8$ and 5 mL of H_2SO_4 (0.3 M). The digested samples were then analyzed by the same equipment (Skalar 5100, Skalar Analytical, Netherlands).This analysis was performed in duplicate.

2.2.2.5. Exopolysaccharide quantification

For EPS quantification, the cell-free supernatant, which contained the polymer, was dialyzed using a 12000 MWCO membrane (Roth Nalo Cellulose Membrane SO farblos) against deionized water, with constant stirring. The water was changed frequently and the conductivity was measured throughout the dialysis process, until a value bellow 10 µS m^{-1} was reached (around 48 h). Sodium azide, at a concentration of 10 $mg.L^{-1}$, was added to prevent possible biological degradation of the polysaccharide during the dialysis process.

Afterwards, the purified polymer was freeze dried (Scanvac, CoolSafe) for 48 hours, and weighed, allowing the determination of the polysaccharide content during the cultivation run.

2.2.3. Calculus

2.2.3.1. Product yield

The product yield on substrate ($Y_{p/S}$,$g.g^{-1}$) was determined as follows:

$$Y_{p/S} = \frac{\Delta p}{\Delta S}$$

where Δp is the the product produced (g_{EPS}) and ΔS is the substrate consumed ($g_{glycerol}$) during the cultivation run.

2.2.3.2. Volumetric productivity

The volumetric productivity (r_P, $g.L^{-1}.d^{-1}$) of the EPS production process was determined using the following equation:

$$r_P = \frac{dP}{dt}$$

where dP corresponds to the variation of concentration of product (EPS, g. L^{-1}) in a dt interval (days), that corresponds to the duration of the production assay.

2.2.4. Biopolymer characterization

2.2.4.1. Sugar and acyl groups

The biopolymer was analyzed in terms of sugar monomers and acyl groups composition. For determination of the sugar composition, dried samples of exopolysaccharide (~5 mg) were dissolved in deionized water (5 mL) and hydrolyzed with trifluoroacetic acid (TFA) (0.1 mL TFA 99%), at 120 ºC, for 2 hours. The hydrolysate was used for the identification and quantification of the constituent monosaccharides by liquid chromatography (HPLC) using a CarboPac PA10 column (Dionex), equipped with an amperometric detector, as described by (Freitas et al., 2014). The analysis was performed at 30 ºC with sodium hydroxide (NaOH 4 mM) as eluent, at a flow rate of 0.9 $mL.min^{-1}$. D-(+)-Galactose (99%, Fluka), D-(+)-glucose anidra (99%, Scharlau), D-(+)-fucose (98%, Sigma), D-(+)-Xylose (99%, Merck), L-rhamnose monohydrate (99%, Fluka), D-(+)-mannose (99%, Fluka), D-glucuronic acid (98%, Alfa Aesan) and D-(+)-galacturonic acid monohydrate (97%, Fluka) were used as standards (50 – 1 ppm).

The acid hydrolysates were also used for the identification and quantification of acyl groups. The analysis was performed by HPLC with and Aminex HPX-87H 300×7.8mm (Biorad), coupled to an infrared (IR) detector, using sulphuric acid (H_2SO_4 0.01 N) as eluent, at a flow rate of 0.6 $mL.min^{-1}$ and a temperature of 30 ºC. Standard solutions of acetate (99%, Fischer Chemicals), succinate (99%, Merck) and pyruvate (99%, Sigma-Aldrich) were also prepared (1 – 100 ppm).

This analysis was performed in duplicate.

2.2.4.2. Molecular mass distribution

Number and average molecular weights (M_n and M_w, respectively), as well as the polydispersity index (M_n/M_w), were obtained by size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS), as described by Freitas et al. (2014). Briefly, FucoPol solutions (2 $g.L^{-1}$) were dissolved in 0.1 M Tris-HCl, NaCl (0.2 M), pH 8.1 buffer, which was also the SEC mobile phase. The SEC colums (PL aquagel-OH mixed 8 μm, 30 x 7.5 mm) were equilibrated for 24 h before running the analysis at a flow rate of 0.7 ml/min at room temperature. In order to follow the purity and molecular mass distribution of the polysaccharide signals from MALLS were recorded in parallel and treated with Astra (V 4.73.04). A d*n*/dc of 0.190 $mL.g^{-1}$ was assumed to calculate the M_w of FucoPol.

2.3. Results and discussion

Nutritional conditions, such as phosphorus concentration, have a large impact in the yield of the production process and may also affect exopolysaccharide composition. Moreover, a reduction of the phosphorus concentration in the medium will also contribute to a decrease in the production costs. As so, this study aimed to assess the impact of phosphorus concentration on FucoPol production, as well as on the polymer's properties.

2.3.1. Effect of phosphorus in exopolysaccharide synthesis

Phosphorus is present in the cultivation medium E* as the following phosphate salts: $(NH_4)_2HPO_4$, K_2HPO_4 and KH_2PO_4. $(NH_4)_2HPO_4$ was kept constant in all assays, since it served as the nitrogen source, and the different phosphorus concentrations in the medium were achieved by altering the content of the other two salts.

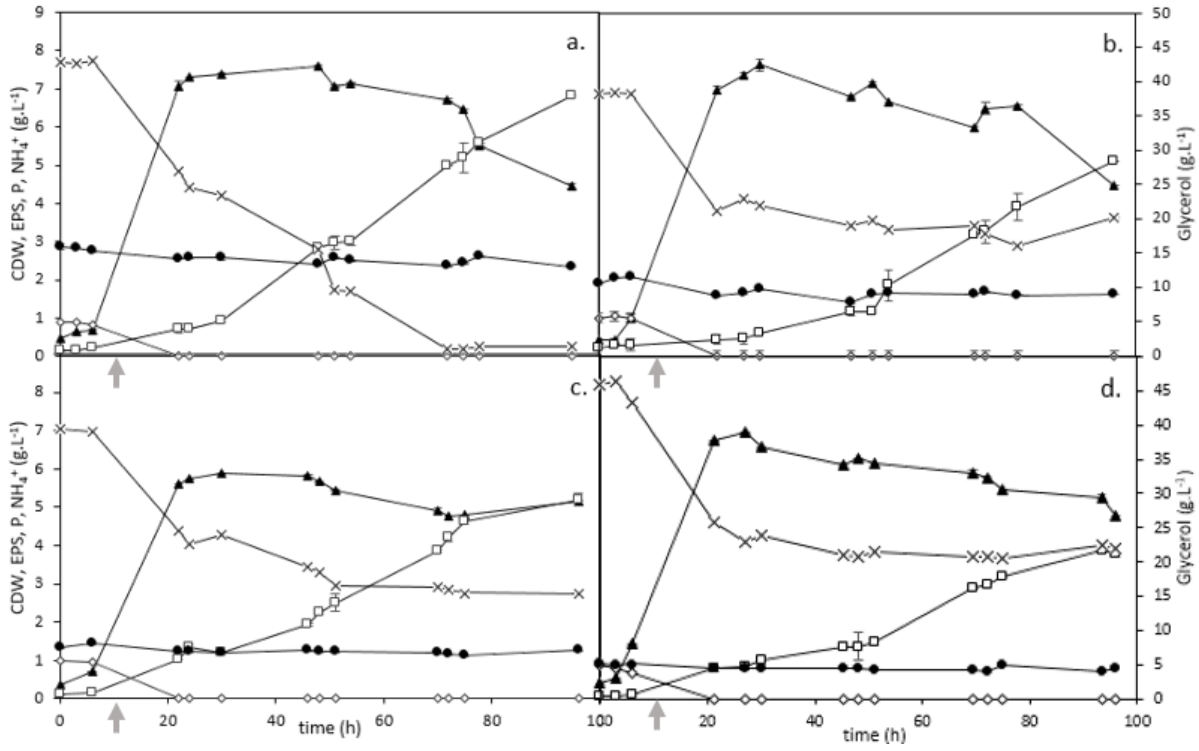

Figure 2.1 – Cultivation profile of *Enterobacter* A47 with different phosphorus concentrations: a. 2.6 g.L^{-1}, b. 2.0 g.L^{-1}, c. 1.3 g.L^{-1} and d. 0.8 g.L^{-1}; wherein experimental results of CDW (▲), EPS (□), ammonia (◊), phosphorus (●) and glycerol (×) are represented throughout the cultivations run. The fed-batch phase was initiated after 10 h of cultivation (⇑).

Figure 2.1 shows the cultivation profiles of *Enterobacter* A47 when different phosphorus concentrations were present in the cultivation medium. Besides the standard phosphorus concentration, 2.6 $g.L^{-1}$ (P1 – Fig. 2.1 a.), three other conditions phosphorus concentrations were studied: 2.0 $g.L^{-1}$ (P2 – Fig. 2.1 b.), 1.3 $g.L^{-1}$ (P3 – Fig. 2.1 c.) and 0.8 $g.L^{-1}$ (P4 – Fig. 2.1 d.). The lowest phosphorus concentration studied (0.8 $g.L^{-1}$) corresponded to the medium's content in $(NH_4)_2HPO_4$ that was used as both phosphorus and nitrogen source.

Standard cultivation assay

Fig. 2.1 a. presents the concentration profiles of biomass, EPS, ammonia, phosphorus and glycerol, for the cultivation of *Enterobacter* A47 with the standard phosphorus concentration in the medium. The exopolysaccharide-producing culture grew exponentially in the first day of the run (Fig. 2.1 a), reaching a maximum CDW of 7.59 $g.L^{-1}$ (Table 2.1). This value is within the range of those reported in literature for the same cultivation conditions.

After 10 h, the bioreactor was operated in a fed-batch mode, by feeding the culture with a mineral solution supplemented with glycerol, at a constant flow rate of 5 $mL.h^{-1}$. It is known that FucoPol production is partially growth associated (Torres et al., 2011) and is enhanced in the stationary phase, with low nitrogen and oxygen conditions, concomitant with carbon source, glycerol, availability (Torres et al., 2014). As so, the feeding solution was rich in glycerol (200 $g.L^{-1}$) and the ammonia was in limiting concentrations (0.9 $g.L^{-1}$). At this stage, the culture was in the stationary phase, where no growth was verified due to nitrogen and oxygen limitation imposed. An apparent decrease in the CDW was observed 48 h into the cultivation run, which was probably due to a dilution of the biomass content associated with the loss of cells by sampling, concomitant with the inflowing of the feeding medium and pH control solutions. Moreover, the cultivation broth became increasingly viscous throughout the run, which made the measurement of the optical density less accurate and, consequently, the CDW calculation was also less accurate.

Simultaneously with bacterial growth, glycerol concentration decreased rapidly in the first 24 hours (from 42.81 $g.L^{-1}$ to 24.62 $g.L^{-1}$) (Fig. 2.1 a). Afterwards, glycerol was added into the bioreactor in the feeding solution (200 $g.L^{-1}$, 5 $ml.h^{-1}$) but was still being consumed by the culture, even though the cells weren't multiplying. At this stage, the cells were using the substrate mainly to synthesize EPS. From the third day on (72 h), glycerol concentration was maintained below 2 $g.L^{-1}$, showing that all the substrate fed to the bioreactor was being consumed by the bacterial cells (Fig. 2.1 a).

EPS synthesis was initiated in the batch phase, as shown by a concentration of 0.23 $g.L^{-1}$, detected at 6 h of cultivation (Fig. 2.1 a). EPS production improved greatly in the stationary phase.

Indeed, after 24 h, 0.72 $g.L^{-1}$ of exopolysaccharide were already produced. At the end of the cultivation run, *Enterobacter* A47 had produced 6.80 $g.L^{-1}$ of biopolymer. Concomitant with EPS production, there was a gradual increase of the apparent viscosity of the broth from 0.02 to 0.91 Pa/s (measured at 0.82 s^{-1}) throughout the cultivation run. It is noted that the apparent viscosity of the broth increased two orders of magnitude (10^{-2} to 10^{0}), resembling the results reported in previous studies (Alves et al., 2010b; Torres et al., 2011). This alteration in the rheology of the culture broth is probably due to the accumulation of the EPS in the cultivation medium (Alves et al., 2010b). However, the increase in viscosity compromised the optical density measurement, which could also explain the apparent reduction of the CDW.

Table 2.1 –Parameters of exopolysaccharide production by *Enterobacter* A47 with different phosphorus concentrations in the cultivation medium.

	literature [2]	***P1***	***P2***	***P3***	***P4***
$P\ (g.L^{-1})$	2.6	2.6	2.0	1.3	0.8
$CDW_{max.}\ (g.L^{-1})$	5.70 – 7.68	7.59	7.64	5.89	7.01
$EPS_{\ produced.}\ (g.L^{-1})$	7.23 – 7.97	6.80	5.12	5.20	3.84
$P_{\ consumed}\ (g.L^{-1})$	-	0.98	0.65	0.41	0.33
$P_{\ biomass}\ (\%)$	-	1.4	1.4	1.4	1.3
$r_P\ (g.L^{-1}.d^{-1})$	1.89 – 2.04	1.72	1.28	1.30	0.96
$Y_{P/S}\ (g.g^{-1})$	0.10 – 0.17	0.10	0.10	0.10	0.07

As can be seen in table 2.1, the amount of EPS produced was slightly lower than the obtained in previous studies, which could be due to differences in the quantification methods. In this work a higher dilution (1:10 instead of 1:2) was used in the extraction of the EPS from the broth, which could have increased the error in the quantification.

Considering 95 h the time frame of the cultivation assay, a volumetric productivity of 1.72 $g_{EPS}.L^{-1}.d^{-1}$ was achieved (table 2.1). These values, although slightly lower, are close to the ones reported in the literature, 1.89-2.04 $g_{EPS}.L^{-1}.d^{-1}$ (Freitas et al., 2014; Torres et al., 2011, 2012 and 2014). The yield of EPS on glycerol in this cultivation run was 0.10 $g_{EPS}.g_{glycerol}$, which is in the range of the values reported in literature for the same cultivation conditions (table 2.1).

[2] The values shown in table 2.1 from literature were obtain from Freitas et al. (2014), Torres et al. (2011), Torres et al. (2012) and Torres et al. (2014).

During the batch phase, the phosphorus concentration in the cultivation broth decreased, concomitant with cell growth, but it remained practically constant (~2.5 $g.L^{-1}$) during the fed-batch phase (Fig. 2.1 a). This result shows that practically all the phosphorus provided by the feeding was consumed by the culture. There was an overall phosphorus consumption of 0.98 g.L-1 (Table 2.1). Hereupon, it is apparent that this nutrient was required not only for the metabolism involved in cell growth, as expected, but also for EPS synthesis, since it continued to be consumed during the fed-batch phase when cell growth was restricted. For many gram-negative bacteria (e.g. *Azotobacter vinelandii*, *Pseudomonas* NCIB 11264, *Klebsiella aerogenes*), studies proved that the enzymes responsible for the synthesis of the precursors involved in EPS formation are constitutive (Horan et al., 1981). Moreover, Sutherland (1982) proposed that maybe some precursors and enzymes required for EPS synthesis are also used during growth for the synthesis of other compounds (e.g. UDP-galactose), such as wall polymers, teichoic acids or lipopolysaccharides. The author also refers the anabolism of glucose as an example, where glucose-1-phosphate is synthesized and can be converted to UDP-glucose, a precursor used in the EPS biosynthesis.

Different phosphorus concentration assays

In this set of experiments, different phosphorus concentrations were tested for *Enterobacter* A47 cultivation and EPS production.

In all assays, the culture reached maximal biomass concentration around the first day of cultivation (Fig. 2.1 b, c and d), with CDW values between 5.89 and 7.59 $g.L^{-1}$ (table 2.1). Hereupon, it is possible to presume that the phosphorus concentration in the cultivation medium had no significant effect on the cell growth of *Enterobacter* A47 in the range of concentrations tested. The fed-batch phase was in all experiments initiated within 10 hours of cultivation. The ammonia present in the medium was completely consumed in all assays and kept bellow the detection limit until the end of the cultivation runs (Fig. 2.1 b, c and d).

In terms of exopolysaccharide production and glycerol consumption, major changes in the cultivation profile occurred when different phosphorus concentrations were present in the medium. In contrast with run P1, glycerol concentration was kept high (above 15 $g.L^{-1}$) during the fed-batch phase of runs P2, P3 and P4 (Fig. 2.1). In fact, when compared with the results obtained from the standard assay (run P1), in which 67.50 $g.L^{-1}$ of glycerol were consumed, less glycerol (51.3-56.7 $g.L^{-1}$) was consumed in all of the other conditions tested (runs P2, P3 and P4).

Concomitant with this reduction in the consumption of substrate, there was also a lower EPS production (table 2.1). Reducing the phosphorus concentration in the medium to 2.0 and 1.3 $g.L^{-}$

^{1} (runs P2 and P3, respectively) resulted in an EPS production of only 5.12 and 5.20 $g.L^{-1}$ (i.e., there was a reduction of approximately 24% compared to the production of run P1). Reducing the phosphorus concentration in the medium to 0.8 $g.L^{-1}$ in run P4, led to a further reduction of EPS production to only 3.84 $g.L^{-1}$ (Table 2.1), which represents a reduction of around 44% compared to the standard conditions of run P1.

Correspondingly, the overall volumetric productivity of runs P2 and P3 were similar (1.28 $g_{EPS}.L^{-1}.d^{-1}$ and 1.30 $g_{EPS}.L^{-1}.d^{-1}$, respectively), while that of run P4 was considerably lower (0.96 $g_{EPS}.L^{-1}.d^{-1}$) (table 2.1). Runs P2 and P3 also presented the same product-substrate yield, 0.10 $g_{EPS}.g_{glycerol}^{-1}$, similarly to run P1. On the contrary, for run P4, a lower yield of 0.07 $g_{EPS}.L^{-1}.d^{-1}$ was obtained.

The results achieved in this study seem to indicate that the phosphorus concentration in the cultivation medium of *Enterobacter* A47, although not having an impact on cell growth, significantly affected the production of exopolysaccharide. This impact was more pronounced for the lowest P concentration tested, for which polymer production, overall volumetric productivity and product-substrate yield were lower than the values obtained under the high phosphorus concentration conditions of run P1.

As can be seen in figure 2.1, independently of the initial phosphorus concentration in the medium, in all assays, it tended to decrease during the batch phase, remaining practically constant during the fed-batch phase. However, the overall phosphorus consumption was reduced (table 2.1). In fact, the highest consumption value (0.98 $g.L^{-1}$) was achieved for the phosphorus rich medium of run P1, being gradually reduced for runs P2, P3 and P4 (table 2.1). This result might be related to the lower EPS synthesis observed as the P concentration was decreased. Polysaccharide synthesis in bacteria requires the biosynthesis of activated precursors, nucleoside diphosphate sugars (NDP-sugars), which are derived from phosphorylated sugars (Freitas et al., 2011a). Therefore, the decreasing phosphorus consumption observed in runs P2, P3 and P4 suggests the lower requirement of this nutrient for the biosynthesis of the EPS building blocks.

There are two transporter systems responsible for the uptake of Pi by the cell: the constitutive Pit system and the Pst system, which is activated by low P concentration. Kinetically, Pst is a high affinity but low velocity system, since it transports this nutrient against the concentration gradient (Jansson, 1988). As so, in phosphorus limiting conditions, Pst is the more efficient transporter of P into the cells (Rao and Torriani, 1990), although at a lower rate than the system Pit that is the main transporter of phosphorus (low affinity, high velocity system). For all the concentrations tested, the Pit system was accounted for the majority of the phosphorus uptake, since the specific transporter (system Pst) is repressed by P concentrations higher than 0.03 $g.L^{-1}$, according to

Rosenberg et al. (1977). A correlation between the assimilation of Pi and the presence of K^+ has been proposed since potassium is required in the transport of this nutrient (Rosenberg, 1987). In fact, K^+ not only stimulates de assimilation of Pi but also the production of EPS (Sutherland, 1982). In this set of experiments, the reduction of P concentration in the medium was accompanied by a simultaneous reduction of K^+, since K_2HPO_4 and KH_2PO_4 were also the source of potassium. Hence, reducing the concentration of K^+ in the medium could have also contributed for the reduction in the Pi uptake, thus impairing EPS synthesis.

Despite the differences in the overall consumption of this nutrient, the amount of P in the cells was identical (1.3-1.4%) in all conditions tested. As so, *Enterobacter* A47 must have been using this nutrient in its metabolism. Besides, the inorganic phosphate transporter (Pit) is not only responsible for the uptake of P, but also for the release of this nutrient from the cell when the intracellular phosphorus concentration is high, maintaining a stable pool of phosphorus in the cell (Jansson, 1988). This internal Pi-pool then supplies this nutrient to be used by bacteria in its metabolism. Phosphorus is an important metabolic intermediate in several important reactions. For example, it is involved in nucleophilic substitution reactions (Pi), and acts as an electrophile atom in the ATP molecule (Nelson and Cox, 2004). Moreover, the hydrolysis of the ATP molecule provides chemical energy so that many thermodynamic unfavorable reactions can occur, these reactions include the synthesis of precursors and biomolecules, the transport across membranes against the concentration gradient, or mechanical motion (Nelson and Cox, 2004). Also, many pathways are regulated by the phosphorylation of certain enzymes, such as glycolysis, gluconeogenesis, mitochondrial and nucleic acid metabolism (Bergwitz and Jüppner, 2011). Some bacteria are also capable of storing phosphorus in the form of polyphosphate (poly-P) in intracellular vacuoles. Poly P can be used as a supply of P in phosphorus-starvation conditions, or as an energy source, since it can substitute ATP in certain biological reactions. The synthesis of poly-P is related to the rate of phosphorus uptake, since is under control of the same signaling system as the pho regulon (Bergwitz and Jüppner, 2011).

Hereupon, the results seem to indicate that reducing the phosphorus concentration in the cultivation medium had a negative impact on EPS production by *Enterobacter* A47, suggesting that P rich medium favors EPS synthesis.

However, a different trend was documented for other bacteria. Increasing the phosphate in the cultivation medium until a certain value enhanced EPS synthesis, but a further augmentation of this nutrient led to a decrease in the EPS production. Examples include xanthan production by *Xanthomonas campestris*, which was shown to be higher when the phosphorus concentration was between 1.3 and 2.0 $g.L^{-1}$ (Umashankar et al., 1996), and gellan production that was maximized for a P concentration of 1.63 $g.L^{-1}$ (Lee et al., 2009). However, in both cases, the phosphorus

concentration had an impact in cellular growth and the optimal concentration for EPS production did not maximize the growth. As for pullulan synthesis by the fungus *Aureobasidium pullullans*, Yu et al. (2012) concluded that an increase in the phosphorus concentration until 0.53 $g.L^{-1}$ led to an higher EPS production. Higher concentrations of phosphorus did not seem to affect the production of pullulan, which remained maximal in the range tested (0.53 $g.L^{-1}$ to 1.60 $g.L^{-1}$). Moreover, the phosphorus concentration did not affect the growth of *A. pullullans*.

2.3.2. Effect of phosphorus in exopolysaccharide composition and molecular mass distribution

As previously referred, the polymer's chemical composition can change depending on the nutrients present in the cultivation medium. Hence, the EPS obtained in the different experiments of this study were characterized in terms of sugar and acyl groups composition (Table 2.1). Moreover, their average molecular weight (Mw) and polydispersity index (PDI) were also determined (Table 2.1).

Table 2.1 – Physical-chemical characterization of the biopolymers produced by *Enterobacter* A47 with different phosphorus concentrations in the cultivation medium.

	Sugar content (%mol)				***Total acyl Groups (%wt)***	***Mw (x10^6 Da)***	***PDI***
	fucose	***galactose***	***glucose***	***glucuronic acid***			
(Freitas et al., 2011; 2014; Torres et al., 2011, 2012)	30 – 36	22 – 29	25 – 34	9 – 10	12 – 22	4.19 – 5.80	1.30 – 1.44
P1	35	23	31	10	8	4.43	1.91
P2	34	25	30	10	9	3.93	1.58
P3	28	24	39	9	9	8.05	2.78
P4	26	24	41	9	11	7.88	1.99

The glycosyl composition analysis of the exopolysaccharides revealed some differences when *Enterobacter* A47 was grown in media with different phosphorus concentrations. Concerning the standard assay, run P1, the EPS obtained presented the typical FucoPol sugar composition: 35 %mol fucose, 23 %mol galactose, 31 %mol glucose and 10 %mol glucuronic acid (table 2.2). Reducing the P concentration to 2.0 $g.L^{-1}$ (run P2) did not affect the polymer's composition as it had also the expected composition of FucoPol: fucose (34 %mol), galactose (25 %mol), glucose (30 %mol) and glucuronic acid (10 %mol). However, further reducing the P concentration to 1.3 and 0.8 $g.L^{-1}$ (runs P3 and P4, respectively) affected the composition of the polysaccharides synthesized. In fact, the polymers produced in runs P3 and P4 had a higher content of glucose (39-41 %mol) and lower content of fucose (26-28 %mol) than the typical FucoPol (table 2.2).

Regarding the acyl groups' analysis, the results indicated that all the biopolymers had similar acyl groups content (8-11 %wt) (table 2.2). Although slightly lower, these values were closer to the one previously reported for FucoPol (Freitas et al., 2011, 2014; Torres et al., 2011, 2012).

The results shown in table 2.2 also revealed that the polymers produced in run P1 and P2 had an average molecular weight (~4 x 10^6 Da) similar to the values reported in previous studies for FucoPol ($4.19\times10^6 – 5.8\times10^6$). However, when the phosphorus concentration in the medium was reduced in runs P3 and P4, the Mw of the EPS produced by *Enterobacter* A47 increased (~8 x 10^6 Da). The results from the SEC analysis also revealed that the biopolymers were homogeneous, since they all had low polydispersity index values (1.58-2.78).

In terms of the differences in molecular weight, this study is in agreement to what Yu et al. (2012) reported for pullulan production by the fungus *A. pullullans*: an increase in the Mw of the EPS when the phosphorus was reduced. No studies concerning the impact of the phosphorus concentration in the polymer's composition and molecular weight were found in literature.

According to the results, phosphorus seemed not only to have an impact in EPS production and productivity, but also in the physicochemical properties of the final polymer. However, a slight reduction in the phosphorus content from 2.6 to 2.0 $g.L^{-1}$ did not affect the composition and molecular weight of the biopolymer, although a lower production was observed. When the phosphorus concentration was further reduced (runs P3 and P4), the polymer showed a higher glucose and lower fucose contents, and a higher molecular mass value. It is also verified that these different polymers were produced in the assays where a reduction in the phosphorus consumption was noticed, confirming the impact of this nutrient.

Since the synthesis of EPS depends on the availability of the precursors, namely nucleotide diphosphate monossacharides (NDP-sugars), it is possible that the regulation of the EPS biosynthesis relies in the supply of these sugar nucleotides. Some of these compounds are used in

the metabolism of the cell, such as for the synthesis of cellular components (e.g. UDP-glucose), and others are only involved in the EPS biosynthetic process (Sutherland, 1982).

Furthermore, according to figure 1.1 (section 1.3), GDP-fucose and UDP-glucose share the same common precursor, glucose-6-P. Torres et al. (2011) suggested that the decrease in the glucose content is related to the conversion of this sugar into galactose and fucose, possibly due to the fact that the enzymatic machinery required in this metabolic pathway was only available later in the cultivation run. Therefore, maybe the phosphorus concentration has an impact in the pathway that leads to the conversion of glucose into fucose. Maybe the synthesis of a key enzyme of this pathway is under control of a regulation system that uses phosphorus concentration as a signal to the activation/repression of transcription.

2.4. Conclusions

This study showed that the phosphorus content does not have an impact in the growth of *Enterobacter* A47, in the range studied. A reduction in the P added to the medium led to a reduction in production and in the fucose content of the purified polymer. Nonetheless, a reduction in the phosphorus content from 2.6 $g.L^{-1}$ to 2.0 $g.L^{-1}$ can be advantageous, if the reduction in the production's cost overcomes the slight reduction in the FucoPol obtained. Furthermore, the effect of P in the regulation of FucoPol synthesis should be further explored, as for the impact of the potassium concentration in the bioprocess.

3. Evaluation of the flocculating properties of FucoPol

3.1. Introduction

Nowadays, flocculants are widely used in wastewater and drinking-water treatment, food and fermentation downstream processing, as well as in textile, pharmacology and cosmetology industries. They can be classified as inorganic flocculants (e.g. aluminum sulfate and polyaluminium chloride), organic synthetic flocculants (e.g. polyacrylamide derivatives and polyethylene imine) and bioflocculants (e.g. chitosan, sodium alginate and microbial flocculants) (Salehizadeh and Yan, 2014). Synthetic and inorganic flocculants are the most commonly used, due to their lower costs and high efficiency (Giri et al., 2015). However, it was recently discovered that their use raises several health and environmental problems, since they are non-biodegradable and some of their monomers, such as acrylamide, are known to be highly toxic and carcinogenic (Rudén, 2004). Moreover, it was proven that aluminum salts can cause Alzheimer´s disease (Campbell, 2002).

Bioflocculants are macromolecules obtained from natural sources which have the ability to flocculate particles (suspended solids, cells, colloidal solids) out of solution. These flocculants are emerging as an attractive alternative to the traditional flocculants, since they don´t represent a risk to human health, are biodegradable and harmless to the environment.

Although the flocculation mechanism involved in biological systems isn't entirely understood, it´s known that the flocculation process is highly dependent on the physical and chemical characteristics of the flocculant agent. It is believed that biopolymer flocculants promote the aggregation of the particles through one or the combination of these two mechanisms: polymer bridging or charge neutralization. The bridging mechanism proposes an explanation for situations where the flocculant is neutral charged or has the same charge as the particles. In this process, the polymer's chains are responsible for bringing the different particles closer together in order to form flocs. Usually a cation is involved, which reduces the effect of the charges and facilitates the adsorption of the particles by the bioflocculant. In the charge neutralization mechanism, an electrostatic interaction occurs between the opposite charged molecules: the biopolymer and the colloids. The bioflocculant reduces the charge density of the particle surface and, as a result, the repulsion between the colloidal particles decreases, allowing the particles to become closer to each other, encouraging the formation of flocs (Yin et al., 2014; Aljuboori et al., 2015; More et al., 2014).

Over the past few decades, many microorganisms, such as bacteria, algae and fungi, have been reported to produce polymers presenting flocculating capacity. These macromolecules include proteins, glycoproteins and polysaccharides. Microbial biopolymer flocculants have the advantage of being produced economically at large scale, under controlled environmental conditions, and being usually easily recovered from the fermentation broth (Karthiga devi and Natarajan, 2015). Hence, these bio-friendly compounds are attracting great research interest.

Microorganisms, such *Rhodococcus erythropolis* (Peng et al., 2014) and *Bacillus subtilis* (Sathiyanarayanan et al., 2013), produce protein flocculants, whereas bioflocculants produced by *Bacillus licheniformis* (Zhao et al., 2013) and *Halobacillus* sp. (Cosa et al., 2012) are composed of glycoproteins. However, the majority of bioflocculant-producing organisms, such as *Bacillus* sp., *Aeromonas* sp., *Klebsiella* sp. and *Enterobacter* sp., are known to produce polysaccharide flocculants (More et al., 2014; Salehizadeh and Yan, 2014).

Several microorganisms are able to produce carbohydrate polymers with an interesting flocculating activity, and there are few studies where *Enterobacter* sp. is the source of the bioflocculant. Yokoi et al. (1997) reported the potential of a biopolymer produced by *Enterobacter sp.* BY-29 to flocculate not only inorganic suspensions, such as kaolin and active carbon, but also organic suspensions of cellulose and yeast; Lu et al. (2005) proved that the polysaccharide produced by *Enterobacter aerogenes* W-23 could be used to flocculate a trona suspension with higher efficiency than the chemical flocculants usually used. Prasertsan et al. (2006) studied the flocculation rate of the bioflocculants produced by several bacterial strains and concluded that *Enterobacter cloacae* WD7 was the most promising strain. The flocculation activity and mechanism of an EPS produced by *Enterobacter sp.* was also studied by Tang et al. (2014).

Furthermore, the bacterium *Enterobacter* A47 (DSMZ 23139) was previously reported to produce a fucose-containing exopolysaccharide, FucoPol, when using glycerol as a carbon source (Alves et al., 2010b). FucoPol is a high molecular weight (4.19 – 5.80 x 10^6 Da) heteropolysaccharide composed of sugar residues (fucose, galactose, glucose and glucuronic acid) and acyl groups (succinyl, pyruvyl and acetyl) (Torres et al., 2015). These non-carbon substituents grant an anionic character to these macromolecules, creating adsorption sites that allow interactions with ions and other molecules.

The flocculation capacity of FucoPol was previously reported by Freitas et al. (2011b) and was forded evaluated in this study. The flocculation rate was determined using a kaolin suspension and several factors which influence the flocculation ability (pH, temperature, dosage, thermal stability and cation) were investigated and optimized.

3.2. Materials and Methods

The FucoPol used in this study was produced by *Enterobacter* A47 in cultivation run P1 (standard phosphorus concentration), presented in section 2.

3.2.1. Exopolysaccharide extraction and purification

The culture broth recovered at the end of run P1 was diluted with deionized water (1:10, v/v), in order to reduce the viscosity, and the bacterial cells were removed by centrifugation (10375 x g, 45 min, 4 ºC). The cell-free supernatant was then submitted to a thermal treatment (70 ºC, 1 h) to inactivate the bacterial enzymes, preventing a possible degradation of the biopolymer in the subsequent extraction steps. Afterwards, the treated supernatant was centrifuged (10375 x g, 45 min, 4 ºC) to remove precipitated proteins and any remaining cell debris.

In order to reduce the concentration of unwanted components (salts, glycerol, proteins), the treated supernatant was submitted to a continuous diafiltration (constant volume diafiltration) process, using a cross-flow module (Sartocon Slide Holder), equipped with a 100 000 Da nominal molecular weight cut-off (NMWCO) ultrafiltration membrane (Hydrosart® Ultrafiltration Cassette, Sartorius), with a surface area of 100 cm^2, operated at a transmembrane pressure below 1.5 bar. When the EPS solution's conductivity reached values bellow 150 $\mu S\ cm^{-1}$, the membrane module was switched to an ultrafiltration mode (volume reduction) to concentrate the treated supernatant (3:1, v/v). Finally, the solution was freeze dried and the purified biopolymer was stored at room temperature.

3.2.2. Bioflocculant characterization

The sugar and acyl composition, as well as the molecular mass distribution were determined according to the protocol described in section 2.2.4.

3.2.2.1. Inorganic content

In order to quantify the ash content of the polymers, they were subjected to pyrolysis at a temperature of 550 ºC, for 24 hours. The total inorganic content was determined by weighting the polymer before and after the treatment.

3.2.2.2. Protein content

For the determination of the protein content, 5.5 mL samples of aqueous FucoPol solutions (4.5 $g.L^{-1}$) were mixed with 1 mL 20% NaOH and placed at 100 ºC, for 5 min. After cooling on ice, 170 µL of $CuSO_4 \cdot 5H_2O$ (25% w/v) were mixed. The samples were centrifuged (3500×*g*, for 5 min) and the absorbance at 560 nm was measured. Albumin (Sigma-Aldrich) solutions (0.05 – 1.0 $g.L^{-1}$) were prepared and used as standards (calibration curve in appendix 7.2). This analysis was performed in duplicate.

3.2.3. Determination of the flocculation rate

The flocculation rate was measured using kaolin clay as a suspension solid (Sigma-Aldrich, Germany) based in the method previously reported by Li et al. (2008). In brief, 4.9 mL of $CaCl_2$ (249 mmol Ca^{2+} per liter) and 0.1 mL of flocculating agent were added into 45 mL of kaolin suspension (5 $g.L^{-1}$). The mixture was vigorously shaken for 20 s and allowed to stand for 5 min at room temperature. Afterwards, one milliliter was removed from the upper layer of the suspension and its absorbance was measured at 550 nm (OD_{550}), with a spectrophotometer (VWR V-1200, Portugal). For every kaolin suspension prepared and every condition studied, a blank sample was prepared by replacing the flocculating agent by the same volume of deionized water ($OD_{550,blank}$). The flocculation rate was calculated according to the following equation:

$$flocculating\ rate\ (\%) = \frac{OD_{550,blank} - OD_{550}}{OD_{550,blank}} \times 100$$

3.2.4. Flocculation rate of the culture broth and cell-free supernatant

The flocculation rate of *Enterobacter* A47 culture broth and cell-free supernatant were evaluated throughout the cultivation run.

Culture broth samples recovered from the bioreactor throughout the cultivation (0.1 mL) were added to the kaolin suspension and the flocculation rate was determined as described above. Viscous broth samples were diluted (1:10, v/v) prior to the measurement of the flocculation rate. Cell-free supernatant samples, diluted 1:10 (v/v), were also tested using the same procedure.

3.2.5. Effect of different factors in the flocculation rate of FucoPol

In this set of experiments, the purified polymer was dissolved in deionized water and used as a bioflocculant agent. The effect of different bioflocculant concentrations (0.1 – 5 $mg.L^{-1}$), suspended solids content (1 – 10 $g.L^{-1}$) and flocculation time (0 – 30 minutes) on the flocculation rate of FucoPol (1 $mg.L^{-1}$) were evaluated, using a kaolin clay suspension, as described above. The effect of pH (3.4-11.6) and temperature (5-60 ºC) on the flocculation rate of purified FucoPol were also studied. To study the effect of pH, the pH-value of the kaolin suspension was first adjusted using NaOH or HCl (1 $mol.L^{-1}$) and the suspension was then used to determine the flocculation rate. In order to study the effect of temperature, the mixture was kept in a water bath until it reached the desirable temperature and then the flocculation rate was determined, as described above. The thermal stability of FucoPol was examined by measuring the flocculation rate after subjecting a FucoPol solution to different thermal treatments: overnight freezing at -80 ºC, followed by thawing at room temperature; heating at 80 ºC or 100ºC for 2 h; and autoclaving at 120 ºC, 1 bar for 20 min. To test the effect of different metal salts on the flocculation rate, several chloride salts (NaCl, KCl, $MgCl_2$, $FeCl_2$ and $FeCl_3$) replaced $CaCl_2$ at the same concentration (249 mmol ion per liter) and the flocculation rate was measured. All experiments were conducted with 5 replicas.

3.2.6. Scanning electron microscopy (SEM) imaging

Scanning electron microscope was used to observe surface morphology of the kaolin clay particles and kaolin clay particles flocculated with the purified FucoPol. Both particles were obtained by centrifuging (3500 x g, 5 min) the suspensions. The samples were freeze dried and their morphology was assessed through scanning electron microscopy in a Carl Zeiss AURIGA Crossbeam SEM-FIB microscope.

3.3. Results and discussion

3.3.1. Bioflocculant production and characterization

FucoPol was obtained by cultivation of the bacterium *Enterobacter* A47 on mineral medium supplemented with glycerol as sole carbon source, as previously reported in section 2.3.2. (run P1). Figure 3.1 presents the surface morphology of the producing bacterium *Enterobacter* A47 (fig. 3.1 a) and the exopolysaccharide FucoPol (fig 3.1 b).

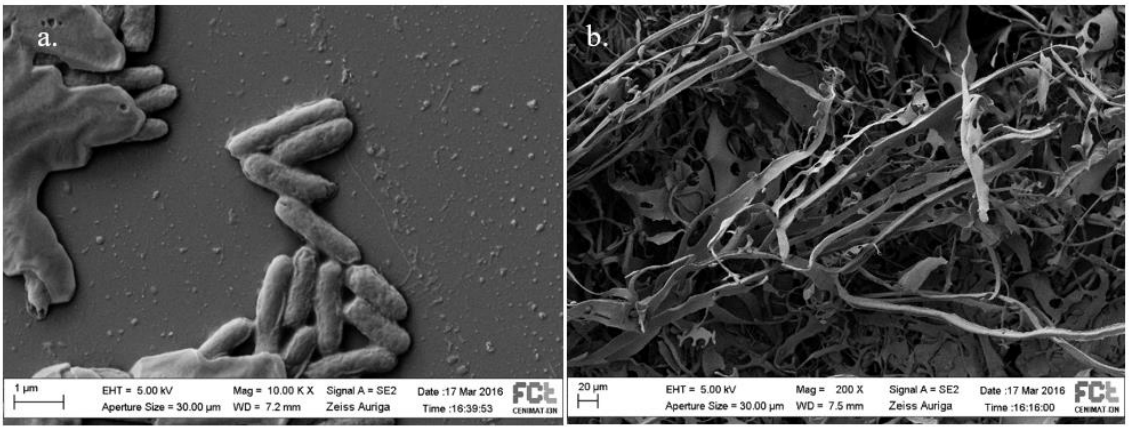

Figure 3.1 – Scanning electron microscopy (SEM) images of (a) Enterobacter A47 and (b) purified FucoPol produced by *Enterobacter* A47.

The glycosyl composition analysis of the purified FucoPol revealed that it was mainly a heteropolysaccharide composed of neutral sugars: fucose (35.4%), glucose (31.1%), galactose (23.4%) and glucuronic acid (10.1%) and containing 8% of acyl groups. After diafiltration process, the purified FucoPol presented a total protein and inorganic salts contents of 10.8 wt% and 7.4 wt%, respectively.

The average molecular weight (M_w) and the polydispersity index (PDI), determined by SEC analysis, revealed that FucoPol is a high molecular weight (4.4×10^6 Da) polysaccharide with low PDI (1.9) showing a homogeneous biopolymer. Envisaging its use as bioflocculant, the high molecular weight of FucoPol is a characteristic that allows higher flocculating ability (Salehizadeh and Yan, 2014).

3.3.2. Evaluation of the flocculation rate of Enterobacter A47 culture broth and cell-free supernatant

The flocculation rate of *Enterobacter* A47 culture broth and cell-free supernatant were evaluated throughout the cultivation run. The results are presented in figure 3.2.

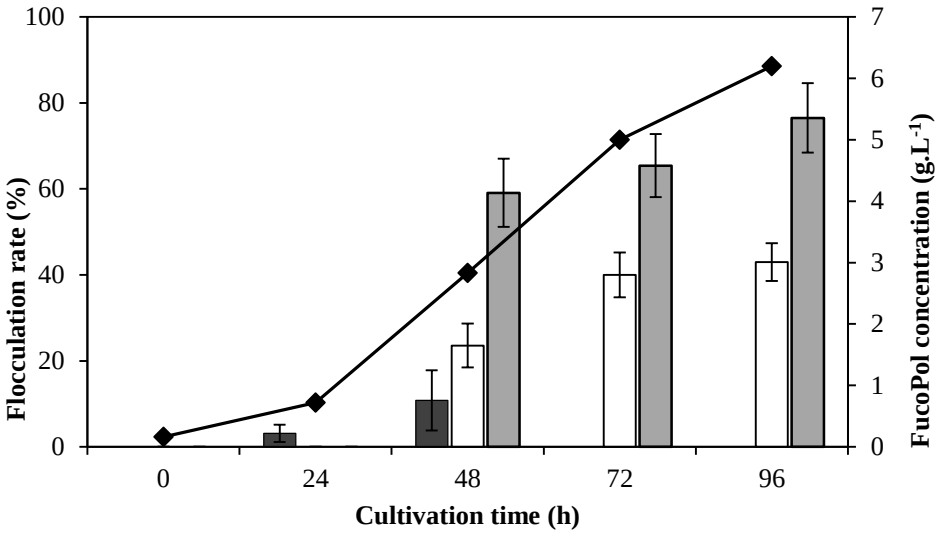

Figure 3.2 – Flocculation rate profile of *Enterobacter* A47 cultivation broth and cell-free supernatant during production of FucoPol (■, Cultivation broth; □, Diluted broth (1:10, v/v); ▭, Cell-free supernatant (diluted 1:10, v/v); ◆, FucoPol concentration).

As shown in figure 3.2, at the beginning of the cultivation it was not detected flocculating capacity, however it can be observed that the flocculation rate increased along the cultivation run as the biopolymer was synthesized. This fact demonstrates that the cultivation medium has no flocculating capacity and the flocculation ability derives from the culture and/or its products. After 24 h of cultivation, a low flocculation capacity was detected which might be related to the presence of FucoPol. At 48 h of cultivation, 2.83 $g.L^{-1}$ of FucoPol had been produced and a flocculation rate of 19% was detected in the culture broth sample. At 72 h of cultivation, no flocculation activity was observed, probably as a result of an increase in the broth's viscosity and/or FucoPol concentration. Since FucoPol provides viscosity to the solutions, high FucoPol concentrations may have created a suspending effect of the particles and the flocculation mechanism was hindered. Concomitant with the bioflocculant production, the broth's viscosity increased from 0.08 to 0.49 Pa/s (measured at 0.82 s^{-1}) between 48 and 72 h of cultivation. Hence,

diluted broth samples were used to measure the flocculation rate in order to confirm whether the higher polymer concentration and/or the increased broth's viscosity was impairing the flocculating capacity of the broth. It can be observed that the flocculation rate of the diluted broth samples increased from 24%, at 48 h of cultivation, to 43%, at the end of the experiment (figure 3.2). Moreover, at 48 h of cultivation, the diluted broth sample with 0.283 $g.L^{-1}$ of FucoPol achieved higher flocculation rate values (24%) than the culture broth sample (19%). A large difference of flocculating capacity between culture broth and diluted broth samples was observed at 72 h of cultivation. At this time, flocculation capacity of culture broth sample was not detected whereas diluted broth sample (1:10, v/v) with 0.5 $g.L^{-1}$ of FucoPol achieved a flocculation rate of 40%. These results suggested that the flocculating capacity was strongly influenced by high concentrations of FucoPol and/or high broth's viscosity.

Cell-free supernatant samples (diluted 1:10 (v/v), as the diluted broth samples) were also tested for their flocculating capacity. The flocculation rates achieved with these samples were strongly higher compared with samples of diluted broth (figure 3.2), suggesting that the cells probably affected the performance of FucoPol as a bioflocculant. At 48 h of cultivation, the flocculating capacity demonstrated by cell-free supernatant samples was much higher (59%) than the values obtained for diluted broth samples. However, for those samples an increase not as significant on the flocculation rates was observed at 72 and 96 h of cultivation. This fact can be related with the high viscosity of the samples that might have interfered with the flocculation mechanism. Nevertheless, along the cultivation run, the flocculating capacity of the cell-free supernatant samples increased, reaching a maximum of 73% at the end of the experiment.

It can be observed that flocculation rate was increased as bioflocculant concentration increased along the cultivation run. The same phenomenon has been reported for several microorganisms, including, for example, *Aspergillus flavus* (Aljuboori et al., 2013) and *Pseudoalteromonas* sp. (Li et. al., 2008), where the maximum of the flocculation rate corresponds to the maximum of bioflocculant concentration.

3.3.3. Evaluation of the flocculation rate of purified FucoPol

The flocculating capacity of the purified polysaccharide synthesized by *Enterobacter* A47 was evaluated by measuring the flocculation rate of a kaolin suspension (5 $g.L^{-1}$) when 0.1 ml of FucoPol (0.5 $g.L^{-1}$) were added. FucoPol concentration used in this study was selected according with the results obtained with the cell-free supernatant samples where higher flocculation rates (59-73%) were achieved using samples (0.1 mL) within a range of 0.3-0.6 $g.L^{-1}$ of FucoPol. As so, the bioflocculant concentration in the falcon was 1 $mg.L^{-1}$.

In order to define the optimum flocculation time to perform the experiments, samples of kaolin clay suspension flocculated with FucoPol and its blank were prepared and allowed to stand for 30 min. During this time, aliquots were taken periodically and the optical density was measured (Figure 3.3).

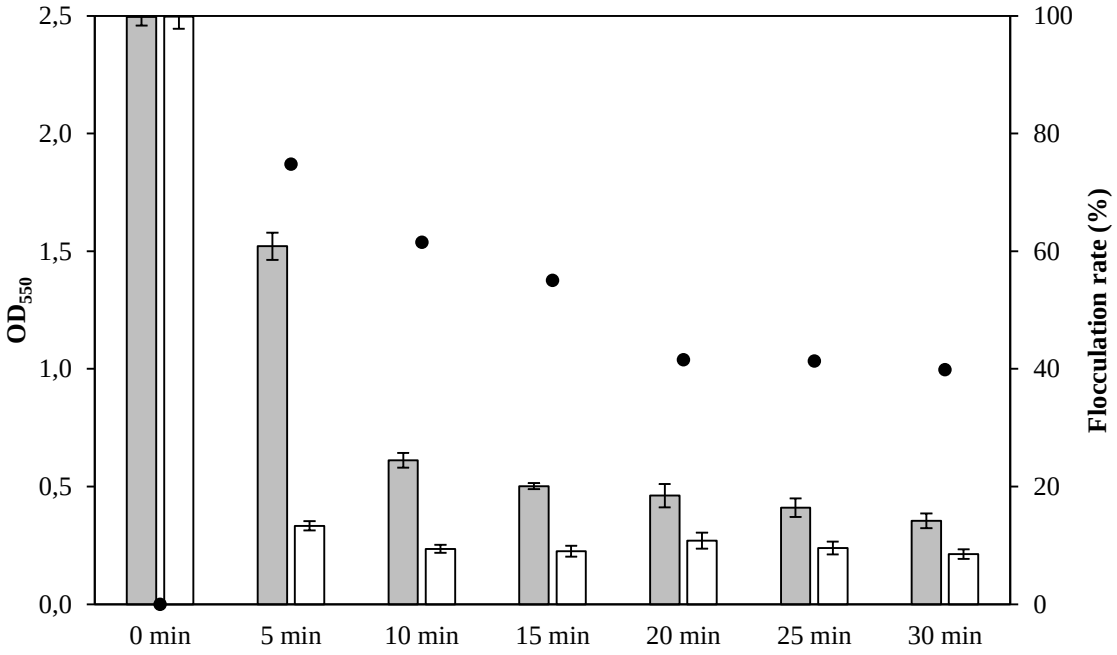

Figure 3.3 – Optical density measured at 550 nm (OD_{550}) and flocculation rate for different settling times (▭, Samples of kaolin clay suspension; □ , Samples of kaolin clay suspension flocculated with FucoPol at a concentration of 1 mg/L; ●, flocculation rate).

As shown in figure 3.3, after five minutes of settling the suspensions presented a completely different behavior. At this time, a strong decreased on the optical density of the samples of kaolin clay suspension flocculated with FucoPol was detected (<0.5) while the value obtained for blank samples remained high (~1.5). Consequently, a sharp rise in the flocculation rate was observed (75%). After 10 minutes of settling, the optical density of the blank samples achieved lower values (0.6) and a decreased on the flocculation rate was notice (62%). As a result of a slight difference between the optical densities of the samples of kaolin clay suspension flocculated with FucoPol and its blank, from 15 minutes to 30 minutes, lower flocculation rates were reached (40-55%). On the basis of this result, for the following experiments, a flocculation time of 5 min was chosen for the flocculation rate measurements.

The surface morphology of the purified FucoPol, the kaolin clay particles and the kaolin clay particles flocculated with FucoPol was observed under scanning electron microscopy (SEM) (figure 3.4). As shown in figure 3.4a, the purified FucoPol exhibits a fibrous structure with spaces

between the fibers network. Figures 3.4b and 3.4c show the morphology of the kaolin clay particles and the kaolin clay particles flocculated with FucoPol, respectively. It can be observed that kaolin clay particles presented low dimensions and were distributed homogeneously for all the area while the particles that were flocculated with FucoPol tend to aggregate and consequently, presented high dimensions. The agglomerates observed in Figure 3.4 c show the ability of FucoPol to flocculate kaolin clay particles.

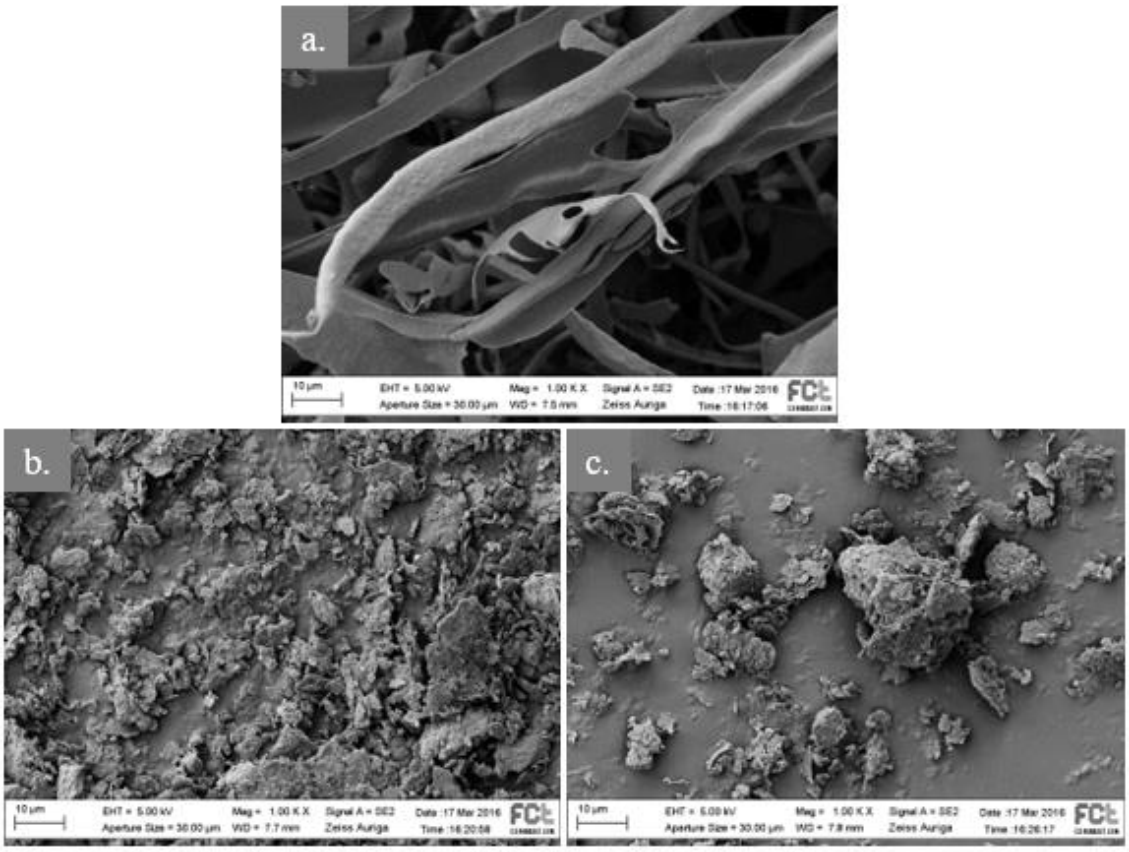

Figure 3.4 – Scanning electron micrograph of (a.) purified FucoPol (b.) kaolin clay particles and (c.) kaolin clay particles flocculated with purified FucoPol (1 mg.L^{-1}) produced by *Enterobacter* A47.

3.3.4. Effect of bioflocculant dosage and suspended solids content

The effect of different bioflocculant dosage and suspended solids content are shown in figure 3.5.

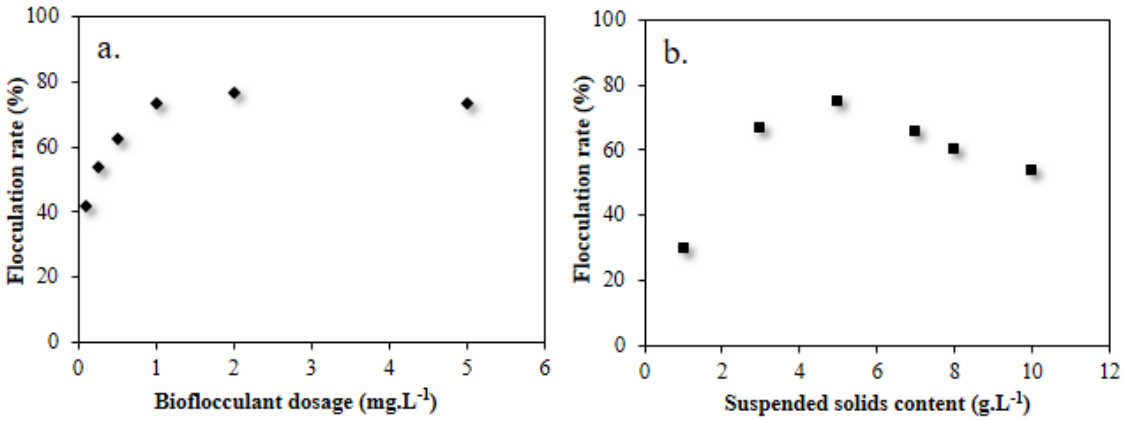

Figure 3.5 – Effect of bioflocculant dosage (a.) and suspended solids content (b.) on the flocculation rate of kaolin clay suspensions using FucoPol as bioflocculant.

Figure 3.5a describes the influence of bioflocculant dosage on the flocculation rate. As expected, low concentrations of bioflocculant (0.1, 0.25 and 0.5 $mg.L^{-1}$) resulted in reduced flocculation rate values (42–63%), which may be related to the fact that the number of bioflocculant molecules was not sufficient to flocculate the suspended kaolin clay particles (Salehizadeh and Yan, 2014). Higher FucoPol dosages, namely, 1, 2 and 5 $mg.L^{-1}$, resulted in flocculation rates of 74–77%. The high molecular weight of FucoPol ($4.4x10^6$ Da) allows that low concentrations of this bioflocculant are able to achieve high flocculation rates since more adsorption points and stronger bridging are created (Salehizadeh and Yan, 2014).

The results obtained are within the range of values reported by Subramaniam et al. (2007) for several microorganisms (71.4–83.7%) such as *Enterobacter* sp. (BS25) where 76.1% of the kaolin clay particles were flocculated using 5 $g.L^{-1}$ of extracellular polymeric substances.

The flocculation properties of FucoPol had already been demonstrated in a previous study (Freitas et al., 2011b) where a FucoPol solution (100 $mg.L^{-1}$) was used to flocculate kaolin clay particles and a flocculating activity of 28% was reached. On that study, the lower value of flocculation rate was probably related with the high concentration of bioflocculant used.

The measured flocculation rate for different suspended solids content is shown in figure 3.5b. The maximum flocculation rate (75%) was reached with 5 $g.L^{-1}$ of kaolin clay suspension that is the concentration commonly used in most flocculation activity studies (More et al., 2014). The flocculation rate decrease (67–54%) as the suspended solids content increase (7–10 $g.L^{-1}$) probably due to the insufficient number of bioflocculant molecules available to flocculate a higher number of kaolin particles.

On the other hand, a lower suspended solids content lead to a reduction in the flocculation rate, which might be due to an inferior number of particles available to form flocs. A different behavior was reported for the bioflocculant produced by *Aspergillus flavus*, since the flocculation rate was constant when the kaolin concentration was in the range of 0.5 to 8 $g.L^{-1}$ (Aljuboori et al., 2015).

As shown in table 3.1, a wide range of bioflocculants can be produced using several carbon sources, with different production efficiencies. The optimal bioflocculant concentration at which the highest flocculating rates were achieved, varied from 1 to 100 $mg.L^{-1}$. Furthermore, it can be observed that bioflocculants produced by different microorganisms are all mainly composed of polysaccharides.

Enterobacter A47 presented the highest flocculant production and FucoPol showed high flocculation efficiency with minimal dosage (table 3.1).

Table 3.1 – Comparison of the flocculation rate for different bioflocculants.

Microorganism	***Carbon Source***	***Bioflocculant production ($g.L^{-1}$)***	***Optimum bioflocculant dosage ($mg.L^{-1}$)***	***Bioflocculant components***	***FR (%)***	***Reference***
Consortium of *Halomonas* sp. and *Micrococcus* sp.	Glucose	3.51	100.0	PS (62.3%), Pr (4.73%) and uronic acid (25.7%)	63.2	Okaiyeto et al., 2013
Aspergillus flavus	Sucrose	N.A.	1.0	PS (69.7%) and Pr (28.5%)	97.4	Aljuboori et al., 2015
Enterobacter sp. ETH-2	Glucose	0.07	1.3	PS (91.7%) and Pr (1.8%)	94.0	Tang et al., 2014
Ochrobactrum ciceri	Corn stover hydrolysate	3.80	N.A.	N.A.	94.0	Wang et al., 2013
Rhodococcus erythropolis	Sludge/livestock	1.6	N.A.	PS (91.2%) and Pr (7.6%)	87.6	Peng et al., 2014
Enterobacter A47	Glycerol	6.80	1.0	PS (81.8%) and Pr (10.8%)	77.0	This study

FR: flocculating rate; N.A.: data not available; PS: polysaccharide; Pr: Protein

3.3.5. Effect of pH and temperature

The effects of pH and temperature on the flocculation rate were studied at a bioflocculant dosage of 1 mg.L^{-1} and kaolin clay suspensions of 5 g.L^{-1}, which were shown to be the most appropriate to reach high flocculation rates with FucoPol as bioflocculant. The experiments concerning the effect of pH on the flocculation were carried out within the range of pH 3.4 – 11.6. Although a maximum was achieved for pH 4.4 (75%), the flocculation rate was relatively stable between pH 3.4 and 5.4 (Figure 3.6a). These results suggest that FucoPol might be a bioflocculant suitable for use in acidic environments, such as wastewater treatment, food and mining industries.

Beyond pH 5.4, the flocculation rate decreased gradually probably due to the different electric states of the bioflocculant at different pH levels, which affects the flocculation ability of the bioflocculant for the kaolin clay particles (Pan et al., 2009). Although there are reports of some pH-resistant bioflocculants, such as, for example, the one produced by *Ochrobactium ciceri* W2 that maintained a high flocculation rate (90%) for pH 1-10 (Wang et al., 2013), most of them are affected by pH in their flocculating capacity. The effect of pH on the flocculation activity of FucoPol was similar to the exopolysaccharide produced by *Gyrodinium impudicum* KG03 that was effective for flocculation of a kaolin clay suspension over a weakly acidic pH range from 3 to 6 (Yim et al., 2007). Aljuboori et al. (2015) also reported the stability of the bioflocculant IH-7 produced by *Aspergillus flavus* at the range of pH from 3 to 7, reaching flocculation activities higher than 90%. Bioflocculant produced by *Kelbsiella* sp. ZZ-3 also presented high flocculation rates between pH 3 to 7 that decreased for higher pH values (Yin et al., 2014).

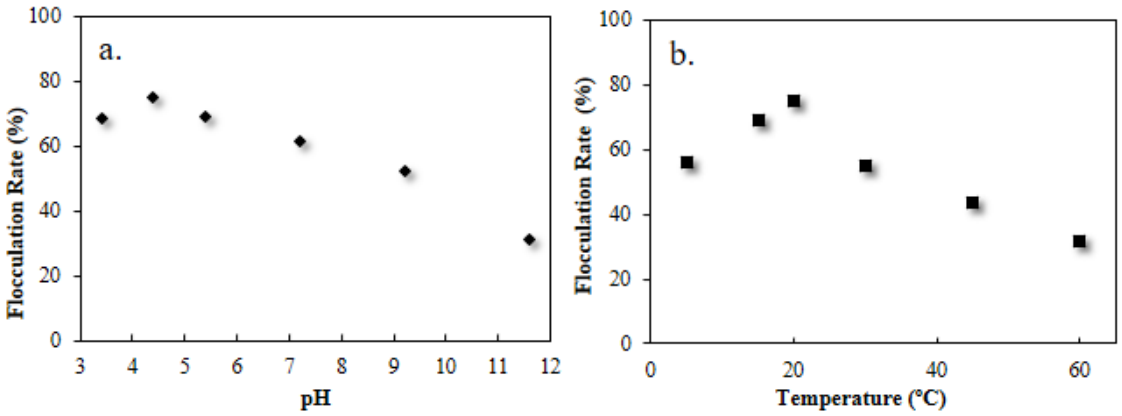

Figure 3.6 – Effect of pH (a.) and temperature (b.) on the flocculation rate of kaolin clay suspensions (5 g.L^{-1}) using FucoPol (1 mg.L^{-1}) as bioflocculant.

In order to investigate the influence of the temperature on the flocculation rate of FucoPol, samples were allowed to stand for 5 min at selected temperatures in a range between 5 and 60 ºC. Figure 3.6b shows that the flocculation rate rose with the increase of temperature up to 20 ºC, where the maximum (75%) was found. However, for higher temperatures (30–60 ºC) the flocculation rate decreased. These phenomena are explained by chemical kinetics since with the increase of temperature the movement of the suspended particles is accelerated, promoting collision and, consequently, the flocculation rate increases. On the other hand, submitting the suspension to higher temperatures can produce smaller flocs with high hydrating trend, decreasing the flocculation rates (Pan et al., 2009). The flocculation ability of FucoPol was higher than that demonstrated by the exopolysaccharide SM9913 produced by *Pseudoalteromonas* sp. SM9913 that had a maximum flocculation rate of 59.25% in a range from 5 to 40ºC (Li et al., 2008).

3.3.6. Thermal stability of the bioflocculant

The thermal stability of the bioflocculant was evaluated by subjecting solutions of purified FucoPol (0.5 $g.L^{-1}$) to different thermal treatments: freezing at -80 ºC and thawing, heating at 80 and 100 ºC, and autoclaving. Each solution was then used to flocculate kaolin clay suspensions (5 g/L).

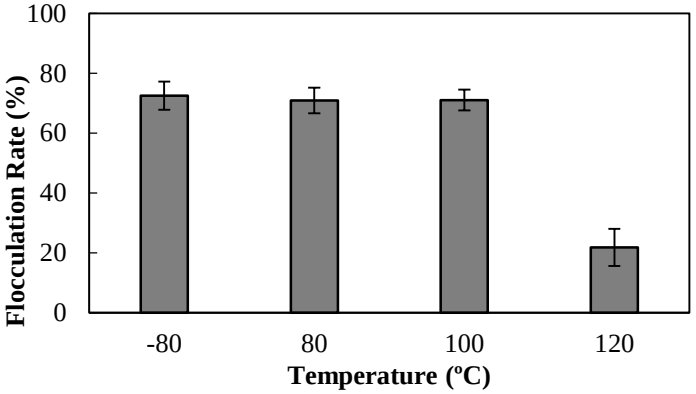

Figure 3.7 – Thermal stability of bioflocculant FucoPol.

As shown in figure 3.7, temperatures as low as -80ºC did not affect the flocculation capability of FucoPol. In fact, after being submitted to freezing and thawing, the biopolymer still presented a 73% flocculation rate. When heated to 80ºC and 100ºC, FucoPol still retained over 70% flocculation rate. As so, FucoPol proved to be thermostable in the temperature range of -80 – 100 ºC. These results were expected since the main backbone of the bioflocculant consists of polysaccharides resulting in higher resistance (More et al., 2014). Previous studies reported the heat stability of several microbial bioflocculants, mainly composed by polysaccharides, in a wide range of temperatures. Bioflocculants produced by *Enterobacter* sp. ETH-2, *Bacillus velezensis* 40B and *Aeromonas* sp. revealed stability in temperatures up to 100 ºC, keeping the flocculation efficiency over 80% (Tang et al., 2014; Zaki et al., 2013; Li et al., 2007).

However, thermal stability of FucoPol was lost when the bioflocculant was submitted to extreme conditions (120ºC, 1bar for 20 min) by autoclaving the solution and the ability of flocculation dropped to only 20%. This thermal behavior might be indicative of an alteration in the conformation of the polysaccharide chain or even a degradation of the bioflocculant (Zaki et al., 2013). The flocculation rate of bioflocculant ZZ-3 produced by *Klebsiella* sp. ZZ-3 also decreased 39 % after being subjected to similar conditions (115 ºC for 20 min) (Yin et al., 2014).

3.3.7. Effect of different cations

As previously described, the flocculation rate of FucoPol was evaluated in the presence of $CaCl_2$. It is known that cations are able to neutralize and stabilize the negative charge of both the kaolin particles and the bioflocculant. Furthermore, cations improve flocculation by forming bridges binding the particles together (Higgings and Novak, 1997; He et al., 2010; Okaiyeto et al., 2013). Also, to several species as *Bacillus*, *Pseudomonas*, *Serratia* and *Yersinia*, the flocculation ability of bioflocculants produced by those microorganisms was improved by the presence of $CaCl_2$ (More et al., 2014).

In order to evaluate the effect of different cations on the flocculation rate of FucoPol, several chloride salts (NaCl, KCl, $MgCl_2$, $FeCl_2$ and $FeCl_3$) were used to replace $CaCl_2$ in the flocculation rate measurement tests. Figure 3.8 shows that the flocculation rate of FucoPol was negatively influenced by the addition of all the other cations tested. Moreover, Na^+, K^+ and Fe^{2+} slightly decreased the flocculation rate, reaching comparable values (49%, 52% and 51%, respectively). Similar results were reported to bioflocculant produced by the consortium of *Halomonas* sp. and *Micrococcus* sp. (Okaiyeto et al., 2013), where the flocculation rate was reduced with the addition of monovalent (Li^+, Na^+ and K^+) and divalent (Mg^{2+}, Mn^{2+} and Ba^{2+}) cations when compared with the flocculating activity observed with Ca^{2+} (75%). On the other hand, the flocculation rate of FucoPol was strongly inhibited by Mg^{2+} and Fe^{3+} and the achieved rates were under 30%. Previous studies also reported the negative impact that Fe^{3+} had on the flocculation ability of bioflocculant produced by other bacteria. For example, the usage of this cation led to a 40% decrease in the flocculation rate of the EPS produced by *Klebsiella* sp. ZZ-3 (Yin et al., 2014) and the bioflocculant produced by *Enterobacter* sp. ETH-2 seemed to lose its flocculation properties in the presence of Fe^{3+} (Tang et al., 2014).

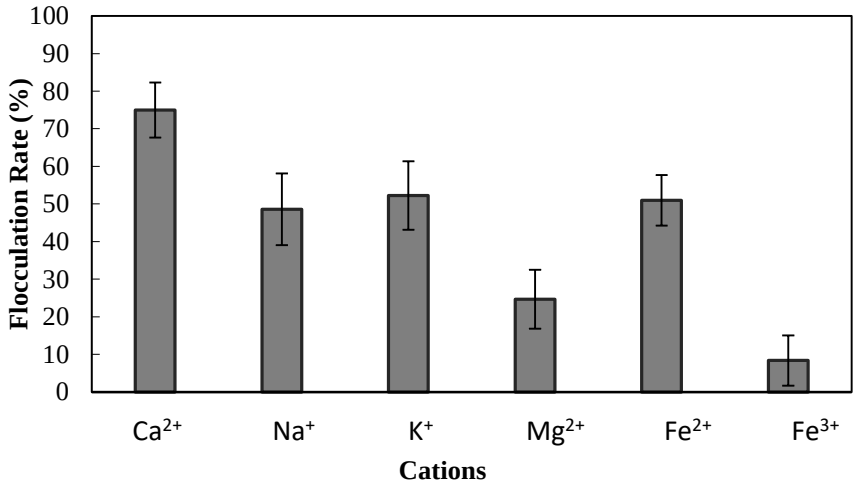

Figure 3.8 – Effect of different cations on flocculation rate of bioflocculant FucoPol.

3.4. Conclusions

Enterobacter A47 produced an exopolysaccharide named FucoPol, which demonstrated flocculation ability. Low concentration of bioflocculant (1 $mg.L^{-1}$) showed a good performance to flocculate kaolin clay particles due to its high molecular weight. Moreover, FucoPol is a polysaccharide, which explains its thermal stability over a wide range of temperatures, suggesting it might be used in both cold and hot conditions. This study revealed that FucoPol has good potential for colloid aggregation in several applications, such as water treatment, food and mining industries.

4. Evaluation of FucoPol's metal binding capacity

4.1. Introduction

Heavy metals are high density metallic substances that are found in trace concentrations in Nature and have a potential adverse effect in the environment and living organisms (Jaiswal et al., 2014). These metals represent a major threat to the environment and human health, since they are non-degradable and extremely toxic at low concentrations. In fact, exposure to these metals affects the nervous system and is associated with the occurrence of numerous cancers, kidney malfunctions and can even cause death (Govind and Madhuri, 2014; Tchounwou et al., 2012).

Despite the fact that these metals have such a negative ecological impact, their use has increased over the past few decades due to industrialization. Industrial operations such as mining, fossil fuel combustion, metallurgy, electroplating, as well as agriculture and domestic activities, have increased the use of metals and chemicals, generating discharged effluents loaded with heavy metals (Kotrba, Mackova and Macek, 2011). These metal discharges into aquatic systems are a major concern since heavy metals are able to accumulate in biological systems: they persist in the organism and accumulate throughout the food chain. Moreover, heavy metals have a high mobility, due to the fact that they are usually present in soluble forms (Govind and Madhuri, 2014).

There are several physical and chemical methods to remove metal ions from aqueous solutions: chemical precipitation, ion exchange, electrochemical treatment, reverse osmosis and chemical extraction. However, these conventional methods are difficult to implement in a large scale, are very expensive and ineffective, especially for concentrations in the range 1 - 100 ppm (Kotrba, Mackova and Macek, 2011; Wang and Chen, 2009).

As an alternative, bio-based methods can be used for wastewater decontamination. These bioremediation methods can either use the living or dead microorganism, or products from their metabolism, such as polysaccharides (Kotrba, Mackova and Macek, 2011). Microbial cells can uptake metals from solution and accumulate these heavy metals bound to the cell wall, reducing their bioavailability. Cellular metabolism can eventually led to the conversion of these metal ions into less soluble and/or toxic forms, by precipitation with metabolites or by redox reactions (Gadd, 2010; Kotrba, Mackova and Macek, 2011). As previously referred, biosorption also occurs in inactive/dead bacterial cells, which indicates that a metabolism-independent process is involved in the association of the metal with the cell. It is believed that this process occurs in a passive way

because the metal cation is able to interact with the acidic functional groups present in cellular structures. Since extracellular polysaccharides are rich in negative charged functional groups (carboxyl, phosphoryl and hydroxyl groups), they play a major role in the biosorption process (Wang and Chen, 2009). When the biosorption is only due to electrostatic interactions or the imbalance of concentrations between the surface of the biosorbent and the solution, the process is considered a physical sorption (Kotrba, Mackova and Macek, 2011). However, other mechanisms could be responsible for the biosorption of metals by a biosorbent, such as microprecipitation, ion exchange, complexation, chelation and coordination (Kotrba, Mackova and Macek, 2011; Wang and Chen, 2009). Microprecipitation occurs when the solubility reaches its limit and the metal precipitates in the surface of the biosorbent. In ion exchange mechanism, a change of ions occurs between the biosorbent and the solution, releasing counter ions (e.g. H^+) into the aqueous solution. When a metal complex is formed due to the association of the metal with another molecule (ligand), the mechanism involved is complexation. When the ligand interacts with the metal through 2 or more sites, it's called chelation, and usually the biosorbent forms a chelation ring attaching the metal. Coordination is a mechanism similar to complexation, but the ligand (biosorbent) binds covalently with the metal (Kotrba, Mackova and Macek, 2011).

Numerous exopolymers obtained from different microbial sources have been shown to be effective in metal sequestration. For instance, exopolysaccharides produced by *Azotobacter chroococcum* (Rasulov et al., 2013), *Paenibacillus jamilae* (Morillo et al., 2006), *Bacillus firmus* (Salehizadeh and Shojaosadati, 2003), *Achromobacter xylosoxidans* (Subudhi et al., 2016), *Herbaspirillium* sp., *Paenibacillus* sp., *Bacillus* sp. and *Halomonas* sp. (Lin and Harichund, 2012) were capable of removing several heavy metals (e.g. Zn^{2+}, Pb^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+} , Co^{2+}, Hg^{2+}) from aqueous systems. Even a consortium of gram-negative bacteria was reported to produce extracellular polymeric substances, which removed over 70% of zinc, lead, chromium, copper, cadmium and cobalt from aqueous solutions (Gawali Ashruta et al., 2014). These results indicate that bacterial exopolysaccharides represent a promising solution for heavy metal sequestration and recuperation from water systems, since they are eco-friendly and biodegradable, and their production is easy and cost effective.

This study focused on evaluating the metal-binding capability of FucoPol, produced by *Enterobacter* A47. The purified biopolymer was tested as a biosorbent of Pb^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+}.

4.2. Materials and Methods

4.2.1. Extraction and characterization of FucoPol

The FucoPol used in this study was produced by *Enterobacter* A47 in cultivation run P1 (standard phosphorus concentration), presented in chapter 2. It was obtained and purified as previously reported in section 3.2.1. The characterization of the biopolymer, in terms of composition and molecular mass distribution, was described in section 3.2.2.

4.2.2. Evaluation of heavy metal binding capacity

To evaluate the ability of FucoPol for binding and removing different heavy metals from aqueous solutions, the method described by Maalej et al. (2015) was performed, with some modifications. Metal solutions (10 $mg.L^{-1}$) of Pb(II), Co(II), Zn(II) and Cu(II) were prepared by dissolving the chloride salts in deionized water: $PbCl_2$ (Sigma, 98%), $CoCl_2.6H_2O$ (Panreac, 98%), $CuCl_2.2H_2O$ (Merck, 99%) and $ZnCl_2$ (Scharlau, 95-100.5%). FucoPol solutions were prepared by dissolving the freeze-dried polymer in deionized water to give different concentrations (10, 50 and 100 $mg.L^{-1}$).

Equilibrium dialysis experiments were carried out by placing dialysis tubes (12-14 kDa MWCO membrane, ZelluTrans Roth) containing 5 mL of FucoPol solution in flasks with 200 mL of the appropriate metal solution. The flasks were placed in an orbital shaker (150 revolutions/min) at 30 ºC, for 24 h. Experiments were performed in duplicates. Controls were performed using deionized water in the dialysis tubing instead of FucoPol.

Afterwards, the content of the dialysis tubing was recovered and all samples were acidified with HCl to prevent metal precipitation. The metallic ions were quantified by Inductively Coupled Plasma-Atomic Emission Spectroscopy (Jobin-Yvon Ultima, Horiba Scientific). The metal adsorbed to the polysaccharide ($M, \mathrm{mg_{metal}}$) and the specific metal uptake ($q, \mathrm{mg_{metal} . g_{EPS}^{-1}}$) were calculated as follows:

$$M = V \; x \; C_{metal}$$

$$q = \frac{V\ x\ C_{metal}}{m_{EPS}}$$

where V is the volume (L) inside the dialysis tubbing, C_{metal} is the metal concentration after equilibrium (mg.L^{-1}) and m_{EPS} represents the mass of polymer (g).

4.2.3. Effect of different factors in lead removal

The effect of polymer dosage on FucoPol metal binding capacity was investigated in a range of 5 – 10000 mg.L^{-1}. Moreover, various initial concentrations of Pb^{2+} solutions (5 – 100 mg.L^{-1}) were tested for a FucoPol concentration of 5 mg.L^{-1}. The effect of the pH value (1.6, 2.3, 3.6 and 4.9) was also tested by adjusting the initial pH of the metal solution with HCl (1.0 M) or NaOH (1.0 M). The impact of temperature (5, 25, 30 and 45 ^{o}C) in the metal removal abilities of FucoPol was also studied. All tests were performed using the procedure described above.

4.3. Results and discussion

Despite the efforts in minimizing the usage of heavy metals, anthropologic activities still generate high amounts of wastewater containing these contaminants. Metals such as zinc, cobalt, lead and copper can cause health problems, as well as environmental damages (Tchounwou et al., 2012). Therefore, there is a growing need for novel, environmentally friendly alternatives to remove heavy metals from contaminated effluents. In this study, the binding efficiency of FucoPol towards heavy metals was investigated.

4.3.1. FucoPol characterization

As previously presented in section 3.3.1, in run P1, *Enterobacter* A47 produced an exopolysaccharide mainly composed of neutral sugars: fucose (35.4%), glucose (31.1%) and galactose (23.4%), and the acidic sugar glucuronic acid (10.1%). The polymer also contained 8% of acyl groups. After purification, FucoPol had a protein content of 10.8% and a total inorganic content of 7.4%. Furthermore, the EPS used in this study was a high molecular weight (4.4×10^6 Da) and homogenous polymer, since it had a low polydispersity index (1.9).

Functional groups with anionic character are known to be responsible for the binding capacity of biosorbents (Gawali Ashruta et al., 2014; Wang and Chen, 2009). Since the EPS produced by *Enterobacter* A47 is rich in these groups, such as such as carboxyl (e.g. in glucuronic acid), hydroxyl (in all the carbohydrates) and acyl groups, the metal sorption potential of FucoPol was further explored in the next sections. The protein content in FucoPol also had functional groups (e.g. amine or carboxyl) that could increase the biosorption of metal by the polymer (Wang and Chen, 2009).

4.3.2. Removal of different heavy metals with FucoPol

The metal-binding efficiency of FucoPol was tested by incubating the exopolymer for 24 hours in solutions containing 10 $mg.L^{-1}$ of different heavy metals: zinc, cobalt, lead and copper. The removal of the cations was quantified in terms of adsorbed cation, *M*, and metal uptake by the EPS, *q*. The results obtained in this set of experiments are presented in figure 4.1. It is also

important to refer that no precipitation occurred in this study, hence, the removal observed was due to the interaction of the polymer with the metal.

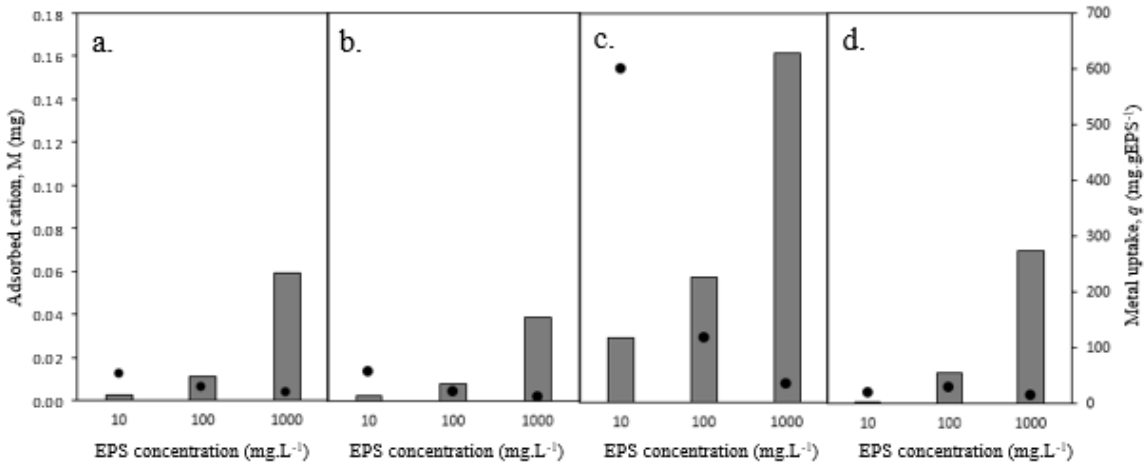

Figure 4.1— Removal of Zn^{2+}(a.), Co^{2+}(b.), Pb^{2+} (c.) and Cu^{2+} (d.) with different FucoPol concentrations. Results are presented in terms of metal uptake (●) and metal absorbed to the biopolymer (□).

As shown in figure 4.1, for all metals, the concentration of FucoPol used had an effect in the metal uptake (*q*), as well as on the total metal adsorbed (*M*). In all experiments, the amount of metal adsorbed (*M*) by the polymer after incubation increased when more FucoPol was present in the dialysis tubing. For all metals, the highest polymer concentration tested, 1000 $mg.L^{-1}$ resulted in the highest *M*.

However, the opposite trend was observed for the specific metal uptake (*q*), in which an increase in the polymer concentration led to a decrease of the *q* values (fig. 4.1). These results could be due to the fact that higher concentrations increased the interactions occurring between the polymer's molecules, which decreased the binding sites available to capture the metals (Salehizadeh and Shojaosadati, 2003). For all metals, with the exception of copper (fig. 4.1 d), the polymer concentration that maximized the specific uptake was 10 $mg.L^{-1}$, decreasing for higher concentrations. Lead presented the most significant difference (fig. 4.1 c), since the metal uptake decreased from 601 $mg.g^{-1}$ to 32 $mg.g^{-1}$, when the concentration of FucoPol rose from 10 to 1000 $mg.L^{-1}$. For copper, similar *q* values were obtained for FucoPol concentrations of 100 $mg.L^{-1}$ (29 $mg.g^{-1}$) and 10 $mg.L^{-1}$ (20 $mg.g^{-1}$).

The results show that FucoPol was able to adsorb all the metallic species tested, however, with different degrees of efficiency. Considering the cation adsorption, *M*, Pb^{2+} was the metal with higher affinity for the exopolysaccharide, since 0.16 mg of this cation were removed with 1000 $mg.L^{-1}$ of EPS. Lower adsorption values were obtained for Cu^{2+}, Zn^{2+} and Co^{2+}, 0.07, 0.06 and

0.04 mg, respectively. Considering the results, the preferential metal adsorption was Pb>>Cu>Zn>Co.

When considering the overall specific metal uptake, Pb^{2+} also presented the highest value, 601 $mg.g^{-1}$ for a FucoPol concentration of 10 $mg.L^{-1}$, while Co^{2+}, Zn^{2+} and Cu^{2+} had considerably lower q values of 53 $mg.g^{-1}$, 48 $mg.g^{-1}$, and 20 $mg.g^{-1}$, respectively. The different removal efficiency observed for the tested metals can be attributed to differences in thecharge density of the ions, which is dependent on the cations ionic size (Salehizadeh and Shojaosadati, 2003). In fact, lead has the higher ionic radius, preceded by cobalt, copper and finally zinc, which was the preferential sequence of specific metal uptake by FucoPol (Pb>>Co>Cu>Zn). Furthermore, according to Salehizadeh and Shojaosadati (2003), the polymer-Pb attraction might have been weaker than with other ions (higher repulsion due to the higher electronic density), and the EPS formed a less compacted structure with the metal. As so, the same amount of lead occupied less surface area and less negative charged functional groups than when the other metals were used. This allowed that more Pb bounded to the EPS after the equilibrium period.

Despite the increase in metal concentration inside the dialysis tubing (M), no insoluble species were formed, which indicated that the polymer interacted with the metals in solution without chemically converting them into less soluble forms. As so, a biosorption mechanism, or a combination of several mechanisms, must be involved in the nature of the FucoPol-metal interaction. Possible interaction mechanisms include microprecipitation, ion exchange, coordination, complexation, adsorption, chelation and electrostatic interactions (Kotrba, Mackova and Macek, 2011; Wang and Chen, 2009), as described before.

The sorption potential of bacterial exopolysaccharides reported in literature showed a wide variety of different results, which could be due to the fact that the interaction between exopolysaccharides and metals highly depends on the structure, composition and surface area of the biosorbent (Rasulov et al., 2013), as well as on the attractive forces and the conformation established between the polymer and the metal (Morillo et al., 2006). Furthermore, differences in the methodologies implemented and in the experimental conditions increased the ambiguity of the results reported in the literature. For instance, Salehizadeh and Shojaosadati (2003) explored the ability of a polymer produced by *Bacillus firmus* to remove Pb^{2+}, Cu^{2+} and Zn^{2+} from an aqueous solution and obtained similar results to those achieved in this study (order of metal uptake: Pb>Cu>Zn). Maalej et al. (2015) tested the metal adsorption properties of an EPS obtained from *Pseudomonas stutzeri* in 10 $mg.L^{-1}$ metal solutions and found that lead had the maximum specific uptake of 251.6 $mg.g^{-1}$, followed by cobalt and copper. That exopolysaccharide did not removed zinc, however. Also, the extracellular polymeric substances

produced by a consortium of gram-negative bacteria adsorbed preferentially zinc, followed by lead, copper and cobalt from aqueous solutions (Gawali Ashruta et al., 2014). Morillo et al. (2006) quantified the metal uptake by an EPS produced by *Paenibacillus jamilae* and reported the following sequence for metal uptake: Pb>>Cu>Zn>>Co.

Despite the differences in the results, it was clear that the exopolysaccharide produced by *Enterobacter* A47 had a good performance for lead sequestration. Therefore, the subsequent experiments focused on the different factors that influenced the adsorption of Pb^{2+} by FucoPol, envisaging the optimization of its use as biosorbent.

4.3.3. Effect of different factors in lead removal by FucoPol

4.3.3.1. Effect of biosorbent dosage

Figure 4.2 describes the effect of the biosorbent dosage on the removal of lead from aqueous solutions.

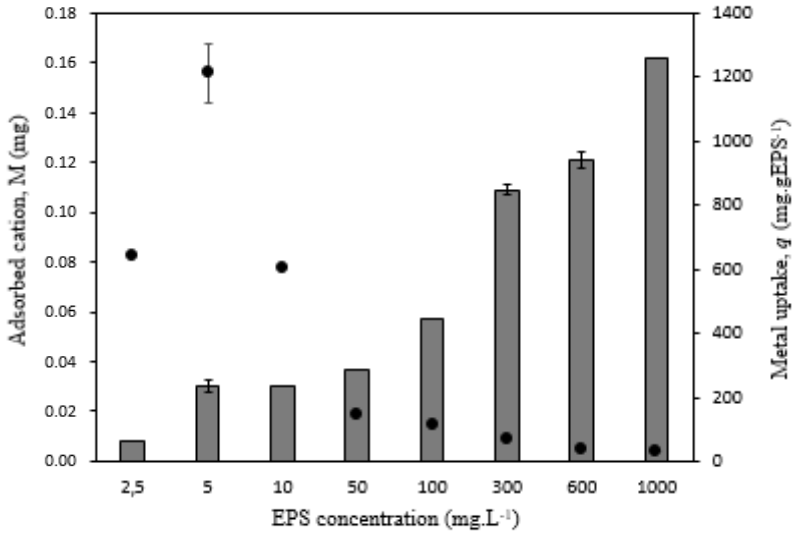

Figure 4.2– Removal of lead with different FucoPol concentrations, for a Pb^{2+} concentration of 10 $mg.L^{-1}$. Results presented in terms of metal uptake (●) and metal absorbed to the biopolymer (▭).

As expected, the metal inside the dialysis tubbing after the incubation period (*M*) increased with the increment of the FucoPol concentration used (fig. 4.2) and maximal sorption was obtained for a concentration of 1000 $mg.L^{-1}$ of FucoPol.

The specific metal uptake (*q*) was maximized (1214 $mg.g^{-1}$) for a FucoPol concentration of 5 $mg.L^{-1}$(fig. 4.2). Increasing FucoPol concentration to 10 $mg.L^{-1}$ resulted in a reduction of *q* to 601 $mg.g^{-1}$, while higher concentrations (50–1000 $mg.L^{-1}$) resulted in a drastic reduction to values below 150 $mg.g^{-1}$. A similar trend was reported for EPS from different bacterial origins. Examples include exopolysaccharides produced by *Pseudomonas aeruginosa* (Gomaa et al., 2012), *Herbaspirillium* spp., *Paenibacillus* sp. and *Pseudomonas* sp. (Lin and Harichund, 2011), where the minimum bioflocculant dosage tested (100 $mg.L^{-1}$) was chosen. However, the EPS produced by *Bacillis firmus* (Salehizadeh and Shojaosadati, 2003) demonstrated an opposite behavior and higher concentrations of biosorbent (from 100 to 1000 $mg.L^{-1}$) improved the lead uptake from ~600 to ~1000 $mg.g^{-1}$.

Figure 4.2 also showed that lowering the polymer concentration (from 5 $mg.L^{-1}$ to 2.5 $mg.L^{-1}$) led to a decrease in the Pb^{2+} uptake. This behavior may be related to the fact that not enough molecules of biosorbent were present in solution and there were not sufficient binding sites to adsorb more lead ions (Ahemad and Kibret, 2013). The studies found in literature did not investigate the impact of biosorbent concentrations lower than 100 $mg.L^{-1}$ in the lead uptake capability of the exopolysaccharides.

As shown in fig. 4.2, the results of *M* and *q* have different profiles for the same conditions. Therefore, in the subsequent studies, the results were presented on specific metal uptake (*q*) since this unit is more frequently used in literature to quantify the metal sorption capability of biopolymers. Moreover, the studies on the effects of metal concentration, pH and temperature in lead removal were carried out with 5 $mg.L^{-1}$ of FucoPol, which was the concentration that maximized *q*.

4.3.3.2. Effect of initial metal concentration

The effect of the lead content in the metal-binding performance of FucoPol was evaluated in a range of 5 to 100 $mg.L^{-1}$, since it was described that the conventional methods are inefficient in removing these concentrations of Pb^{2+} (Kotrba, Mackova and Macek, 2011; Wang and Chen, 2009).

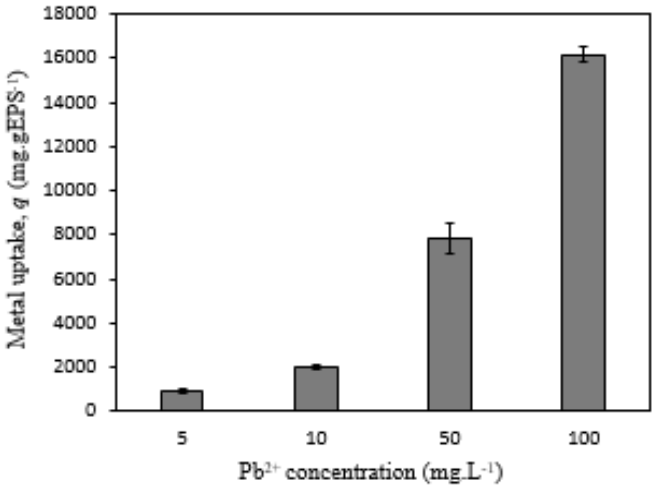

Figure 4.3 – Metal uptake of different initial lead concentrations with 5 mg.L^{-1} FucoPol (temperature 30 ºC; pH 4.9).

The results presented in figure 4.3 proved that the initial concentration of lead had a significant effect on the uptake efficiency of FucoPol. Higher Pb^{2+} concentrations improved greatly the lead uptake by the EPS. In fact, when the concentration of metal was augmented from 5 mg.L^{-1} to 100 mg.L^{1}, the specific Pb^{2+} uptake increased approximately 17 times, reaching a maximum *q* value of 16142 mg.g^{-1}. Due to the differences in methodology, it is difficult to compare data from studies with different polysaccharide biosorbents. Nonetheless, when considering the maximal lead uptake, FucoPol had a much higher efficiency when compared with the values documented for other EPS (table 4.1).

Table 4.1 – Comparison of lead removal efficiency between exopolysaccharides produced by several microorganisms.

EPS producing bacteria	***q (mg.g^{-1})***	***References***
Enterobacter A47	16142	This study
Bacillus firmus	1103	Salehizadeh and Shojaosadati (2003)
Alteromonas macleodii	316	Loaëc et al. (1997)
Paenibacillus jamilae	228	Morillo et al. (2006)
Pseudomonas stutzeri	216	Maalej et al. (2015)
Arthrobacter ps-5	216	Shuhong et al. (2014)
Bacillus sphaeicus	90	Wang et al. (2015)
Azotobacter chroococcum	33.5	Rasulov et al. (2013)

The subsequent studies, namely, the effect of pH and temperature on the metal uptake, were performed with a 100 $mg.L^{-1}$ Pb^{2+} solution and an EPS dosage of 5 $mg.L^{-1}$, which were the most efficient proportions on the removal of lead with FucoPol.

4.3.3.3. Effect of pH in lead removal by FucoPol

Because pH is known to be one of the parameters that highly influences metal biosorption, the Pb^{2+} uptake by FucoPol was evaluated in pH values between 1.6 to 4.9 (figure 4.4). It was not possible to test higher pH-values due to the chemical precipitation of lead that occurred at pH ≥5.6.

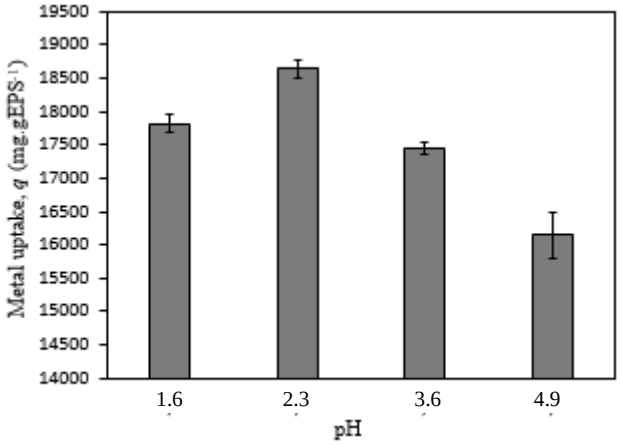

Figure 4.4 – Effect of pH in lead uptake by FucoPol (initial Pb^{2+} concentration of 100 $mg.L^{-1}$; EPS concentration of 5 $mg.L^{-1}$; temperature 30 ºC).

The pH affects the functional groups responsible for the metal-binding activity and the metal solubility (Abbas et al., 2014; Lin and Harichund, 2012). In fact, pH determines the ionization state of important functional groups involved in the metal-binding process, such as phosphate and carboxyl groups (Wang et al., 2015). Depending on the type of EPS-metal interaction, usually acidic conditions tend to decrease the metal uptake, due to the interaction between the negative binding groups and the H^+. Under alkaline conditions, OH^- ions also interfere with the adsorption of metals by the EPS (Lin, 2012).

Figure 4.4 demonstrated that, despite the fact that the pH affected the metal uptake, FucoPol presented an overall high efficiency in binding to Pb^{2+} under acidic conditions (pH 1.6–4.9), reaching a maximal specific metal uptake of 18645 $mg.g^{-1}$ at an optimum pH value of 2.3. These results were slightly different from those previously reported for other polysaccharides.

Biopolymers produced by *Azotobacter chroococcum* XU1 and *Bacillus firmus* demonstrated an optimal lead uptake at pH 4.5 (Rasulov et al., 2013; Salehizadeh and Shojaosadati, 2003), whereas the exopolysaccharide obtained from *Methylobacterium organophilum* was more efficient at neutral pH (Kim et al., 1996). However, optimal pH depends on the mechanisms involved in the biosorption of the metal, as well as the ionic state of functional groups (Kim et al., 1996), which is affected by the chemical environment of these groups (Nelson and Cox, 2004) and, therefore, by the structure of the different polymers.

With FucoPol, further acidic conditions (1.6–2.3) led to a decrease in the metal removal (fig. 4.4). This trend was probably due to the fact that the acidic groups of the polymer were protonated, decreasing the negative charges available to interact with the Pb^{2+} cations (Kim et al., 1996).

Despite lead's nocive impact on the environment and human health, 1.52 million metric tons of this metal are used in the USA for several industries and applications, being the lead-acid batteries manufacturing one of the main activities responsible for such high Pb^{2+} consumption. In fact, this application accounts for 83% of the amount of lead used (Tchounwou et al., 2012). The production of acid lead batteries generates an acid wastewater with a pH between 1.2 and 3.0, to which base is then added to neutralize the pH (Dermentzis et al., 2011). FucoPol would probably be a viable solution to retrieve the lead from these acid waters, since it showed high Pb^{2+} uptake in this range of pH.

4.3.3.4. Effect of temperature in lead removal by FucoPol

Considering that temperature fluctuations occur during the year and that it affects the metal uptake process (Ahemad and Kibret, 2013; Lin and Harichund, 2012), the effect of temperature in the Pb^{2+} removal abilities of FucoPol was explored in a range of 5 ^{o}C to 45 ^{o}C. The results of these tests are presented in figure 4.5.

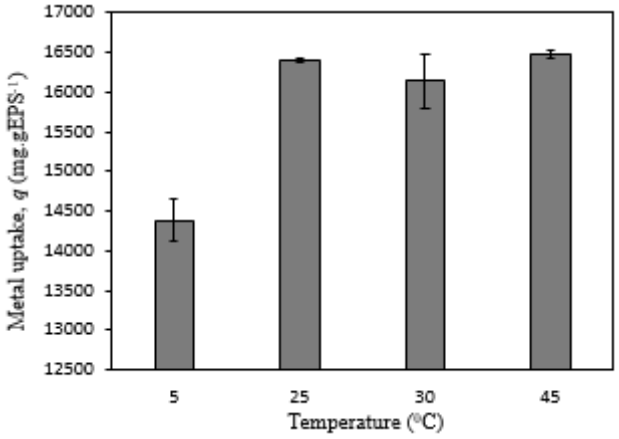

Figure 4.5 – Effect of temperature in the lead uptake by FucoPol (initial Pb^{2+} concentration of 100 $mg.L^{-1}$; EPS concentration of 5 $mg.L^{-1}$; pH 4.9)

As figure 4.5 demonstrates, the lead uptake efficiency of FucoPol was practically constant in a range of temperatures of 25 ºC to 45 ºC (fig. 4.5), meaning that this EPS is thermostable and suitable to be used at these temperatures.

The metal-binding efficiency of FucoPol was slightly affected when low temperatures (5 ºC) were implemented, but the biopolymer still showed a high Pb^{2+} biosorption capacity (14381 $mg.g^{-1}$). When the temperature decreased from 25 ºC to 5 ºC, a reduction in the Pb^{2+} uptake of approximately 12% was verified. According to Wang et al. (2015), this behavior may indicate that biosorption is an endothermic process. The influence of temperature in the specific lead uptake by exopolysaccharides was not investigated in the majority of the metal removal studies. Nevertheless, Lin and Harichund (2012) reported a similar trend for the *Paenibacillus sp.* CH11 biosorbent, since lead removal by this polymer was not affected by temperature in the range from 4 to 45°C. However, this bisorbant showed a different composition, since it has proteins as the major component. Wang et al. (2015) studied the usage of an extracellular polysaccharide, produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6, as a bisorbant of lead, and reported its thermosability within the temperature range of 5 ºC to 35 ºC.

Despite the reduction in metal uptake with lower temperatures, FucoPol proved to be highly efficient in biosorbing lead from aqueous solutions in a range of temperatures studied (5 ºC to 45 ºC). As so, FucoPol could be an effective alternative to be used in the removal of this metal from contaminated wastewaters without temperature control, despite the seasonal variations in this parameter.

4.4. Conclusions

The present study made evident that FucoPol is capable of removing Pb^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+} from aqueous solutions, although with different efficiency. Furthermore, this exopolysaccharide is extremely efficient in the biosorption of lead and was able to remove up to 18645 $mg_{metal}.g^{-1}_{EPS}$. Low EPS concentrations of 5 $mg.L^{-1}$ showed a great performance in binding Pb^{2+} from aqueous solutions. Moreover, FucoPol has proven to be thermostable and suitable to biosorb this metal from acid solutions, suggesting that it can be a viable option to be implemented in wastewater treatment contaminated with lead.

5. Conclusions and future work

In this thesis, the impact of phosphorus concentration on FucoPol production by the bacterium *Enterobacte*r A47 was investigated. Different phosphorus concentrations were provided in the cultivation medium (0.8 to 2.6 $g.L^{-1}$) and the effect of this nutrient was evaluated in bacterial growth, exopolysaccharide production and composition. A reduction in the phosphorus present throughout the cultivation run did not affect cell growth, however it led to a decrease in EPS production, which was more evident for the lowest P concentrations tested (0.8 $g.L^{-1}$). Furthermore, the amount of phosphorus supplied had an impact on the physicochemical properties of the EPS produced at the end of the assays. A slight reduction from 2.6 to 2.0 $g.L^{-1}$ in the phosphorus concentration did not affect the sugar and acyl composition of the EPS, neither the molecular weight of the polymer. However, the polysaccharide obtained with lower phosphorus concentrations (1.3 and 0.8 $g.L^{-1}$) showed a different composition, richer in glucose and with a lower fucose content, and a higher molecular weight when compared with FucoPol.

For future studies, the impact of phosphorus concentration in FucoPol synthesis should be further explored, using higher phosphorus concentrations and/or different P salts. The effect of this nutrient in the regulation of the metabolic pathway should be investigated, by evaluating the enzymatic activity of the enzymes involved in the synthesis of NDP-sugars. K^+ may also be an important factor in FucoPol synthesis so it should be investigated.

In this work, the capacity of flocculation and metal-binding of FucoPol were also accessed to evaluate the polymer's suitability for use in applications such as water treatment, food and mining industries. FucoPol showed a good performance in flocculating kaolin in different suspend solids concentrations, with a flocculation rate up to 75%. Low bioflocculant concentration (1 $mg.L^{-1}$) associated with a cation, preferentially Ca^{2+}, promoted the flocculation of the kaolin particles due to its high molecular weight. Moreover, FucoPol demonstrated to be an efficient flocculation agent under acidic conditions (pH 3.4 to 5.4), suitable to be used in the treatment of wastewaters from food and mining industries. Furthermore, FucoPol did not lose its flocculant properties after being subjected to several thermal treatments, such as freezing (-80^{o}C) and thawing and heating until 100 ^{o}C. The thermostability of the polymer is probably due to its high content in polysaccharides. Now that the optimal flocculation conditions for FucoPol are known, the ability to form colloid aggregations should be tested with other suspended solids in order to find a viable application. The bioflocculant ability of FucoPol could be tested in fermentation broths, soil suspensions and/or food and industry wastewaters.

FucoPol was also capable of removing Pb^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+} from aqueous solutions. In fact, this biosorbent showed excellent results in lead binding, reaching a maximal metal uptake of 18645 $mg.g^{-1}$ for the optimal conditions (initial metal concentration of 100 $mg.L^{-1}$, 5 $mg.L^{-1}$ of FucoPol, pH 2.3). Due to the excellent binding skills under acidic pH, this biosorbent proved to have potential to be used in lead removal from acidic wastewaters, such as the generated by batteries manufacturing. Additionally, FucoPol had high binding activity in a temperature range of 5 to 45 ^{o}C, proving it can be used with high efficiency in wastewater treatment throughout the year, despite the variations in temperature.

In the future, the uptake of other metals, even precious metals, should be further explored in order to optimize the binding conditions. Uptake studies in multi-metal aqueous solutions and in real wastewater samples should be performed. Also, new methodologies should be explored in order to develop an efficient and viable process that could be implemented in large scale wastewater treatment. Furthermore, it should be investigated if the metal recuperation from the polysaccharide is viable, envisaging the reutilization of the metals. This could be particularly useful for precious metals, especially if they were retrieved as nanoparticles.

Hereupon, *Enterobacter* A47 seems to be able to produce exopolysaccharides with different properties, depending on the cultivation conditions. This opens the possibility to design different polymers suitable for several applications, through engineering and manipulation of different environmental factors. The tailoring of the polysaccharide could increase the flocculation rates and metal binding efficiency, getting FucoPol one step closer to be used in the industry or wastewater treatment fields.

6. References

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7. Appendixes

7.1. Glycerol calibration curves

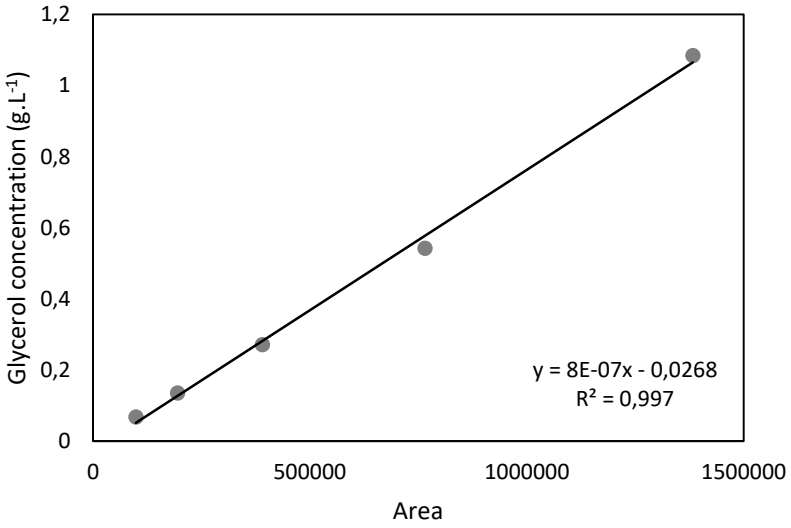

Figure 7.1 – Glycerol calibration curve used in run P1 and P4.

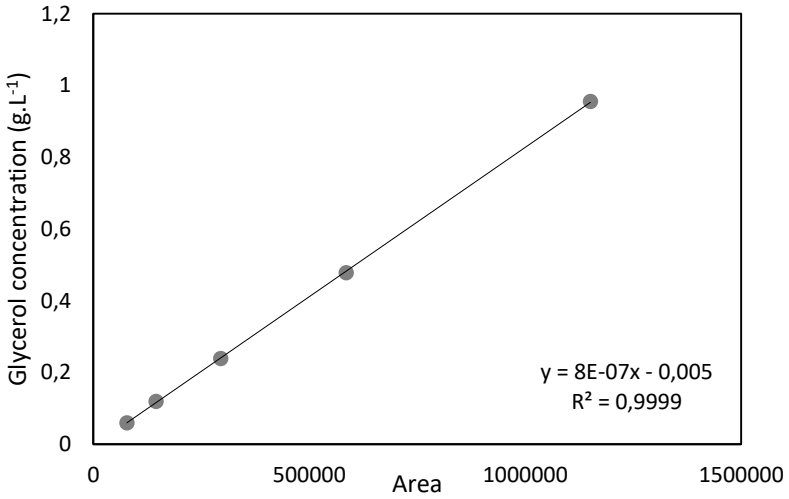

Figure 7.2 – Glycerol calibration curve used in run P2.

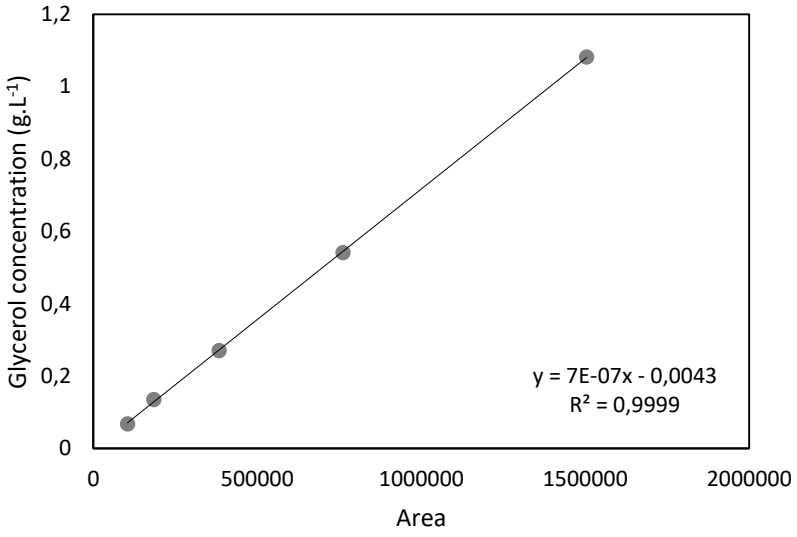

Figure 7.3 – Glycerol calibration curve used in run P3.

7.2. Protein calibration curve

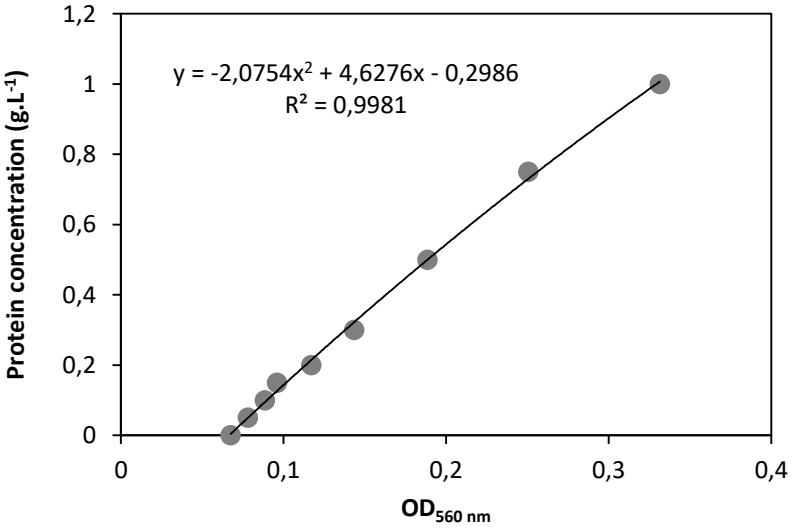

Figure 7.4 – Protein calibration curve.